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THE EFFECTS OF 2',3'-DIDEOXYNUCLEOSIDES ON DUCK HEPATITIS B VIRUS REPLICATION: A MODEL FOR INHIBITION OF HEPADNAVIRUS REPLICATION

BY

WEIXING LUO 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SUBMITTED BY WEIXING LUO

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

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To my husband Xiaoning

and

my parents.

ABSTRACT

I have used a duck hepatitis B virus (DHBV) system to screen compounds for antiviral activity against hepadnaviruses. The results of *in vitro* screening using DHBV-infected duck primary hepatocyte cultures showed that the purine 2',3'-dideoxynucleosides were strong inhibitors of DHBV replication whereas the pyrimidine 2',3'-dideoxynucleosides were not. Among the purine dideoxynucleosides, 2,6-diaminopurine 2',3'-dideoxynucleoside (ddDAPR) and 2',3'-dideoxyguanosine (ddG) were the most effective inhibitors of DHBV. Both had IC_{50} values of 0.07 µg/ml. 2',3'-Dideoxyadenosine (ddA) was also an effective inhibitor with an IC_{50} of 0.12 µg/ml.

The effects of the dideoxynucleosides were examined *in vivo* in DHBV-infected ducks and the results correlated reasonably well with the results obtained *in vitro*. At 10 mg/kg intramuscular (i.m.) twice daily (bid), ddDAPR cleared DHBV DNA from the sera of DHBV-infected ducks within one week. ddA was less effective *in vivo* than it was *in vitro*. The pyrimidine analogue, ddC, was ineffective *in vitro* and showed no inhibitory effect on DHBV replication *in vivo*. These results suggest that *in vitro* screening of antiviral agents in the DHBV system is a reasonable predictor of activity *in vivo*. However, application of results obtained

using the DHBV system to the human hepatitis B virus (HBV) requires further investigation.

The half lives of DHBV DNA in serum and its cccDNA form in liver have been examined *in vivo* in congenitally infected ducks using ddDAPR as an inhibitor to block viral replication. A relatively short half-life of approximately 14 hours was determined for DHBV DNA in serum. The cccDNA form of DHBV in liver, however, is very stable and possesses a relatively long half life. Since cccDNA plays an important role in the maintenance of persistent hepadnavirus infection, this longer half life for cccDNA has important implications for the use of antiviral agents in attempts to "cure" persistent infections.

I have shown that ddGTP, which is assumed to be the active form of ddG (and ddDAPR since this compound is rapidly deaminated to ddG) in cells, showed only a weak inhibitory effect on DHBV DNA polymerase and reverse transcriptase in isolated replicative core particles. These results were strikingly different from the strong inhibition I observed with ddDAPR both *in vitro* and *in vivo*. Therefore, the potent inhibitory activity of ddDAPR on DHBV replication could not be explained by its action on either the DNA polymerase or the reverse transcriptase activities. I have used Southern and Northern blots to examine DHBV DNA and pregenomic RNA in the replicative core particles collected from ducks treated with ddDAPR. The results showed that almost no viral DNA was

synthesized after treatment whereas the amount of pregenomic RNA is only partially reduced. In addition, the replicative core particles isolated after *in vivo* treatment with ddDAPR were unable to incorporate [α^{32}P]-labelled deoxynucleotides indicating inhibition of DHBV DNA synthesis. On the basis of these results, I have proposed that purine 2',3'-dideoxynucleosides bind to the terminal protein to selectively inhibit hepadnavirus replication. This specific site favors dideoxyguanosine analogues since the nucleotide linked directly to the terminal protein is the first dG of the minus strand DNA. Initiation of the minus strand DNA synthesis by dG at the terminal protein appears to be conserved in the hepadnaviruses and could serve as a unique site for specific antiviral therapy.

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
Au Ag	Australian antigen
bid	twice a day
bp	Base pair(s)
BSA	Bovine serum albumin
cccDNA	Covalently closed circular DNA
ddA	2',3'-dideoxyadenosine
ddC	2',3'-dideoxycytidine
ddDAPR	2,6-diaminopurine 2',3'-dideoxynucleoside
ddG	2',3'-dideoxyguanosine
ddI	2',3'-dideoxyinosine
ddT	2',3'-dideoxythymidine
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	2',3'-dideoxyguanosine-5'-triphosphate
DHBV	duck hepatitis B virus
DNase	Deoxyribonuclease
DTT	Dithiothreitol
DR1	direct repeat 1
DR2	direct repeat 2
EDTA	ethylene-diaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetra-acetic acid

FBS	fetal bovine serum
GHBV	ground squirrel hepatitis virus
HBsAg	human hepatitis B surface antigen
HBcAg	human hepatitis B core antigen
HBeAg	human hepatitis B e antigen
HBV	human hepatitis B virus
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IC_{50}	concentration resulting in 50% inhibition
i.m.	intramuscular
kb	kilobases or kilobase pairs
kDa	kilodaltons
MEM	minimum essential medium
min	minute
ml	milliliter
mRNA	messenger RNA
NaOAc	sodium acetate
nm	nanometer
°C	degree Celsius
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
qid	every 6 hours
q8h	every 8 hours
RNase	ribonuclease
RNasin	ribonuclease inhibitor

rpm	revolution per minute
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TAE	Tris-Acetate and EDTA buffer
TBE	Tris-Borate and EDTA buffer
TPDC	terminal protein-DNA complex
WHV	woodchuck hepatitis virus
UV	Ultraviolet
µg	micrograms
µl	microliters
µM	micromolar

CHAPTER 1.

INTRODUCTION

1.1 Hepatitis B Virus Infection and Its Associated Diseases.

1.1.1 Hepatitis B Virus Associated Diseases.

Viral hepatitis attributable to hepatitis B virus (HBV) infection presents a broad spectrum of clinical features. Primary HBV infection may be associated with unapparent or subclinical symptoms (60-70% of cases), and therefore does not come to medical attention. The infection may also be associated with acute hepatitis (20-35% of cases), which varies in severity from mild hepatitis at one extreme to fulminant hepatitis at the other extreme (Hollinger, 1985, Gitnick, 1989, Koff, 1989). About 90% of adult patients will recover completely from an infection with HBV. However, 5-10% of patients who suffer with clinical hepatitis or even subclinical symptoms do not recover normally but become chronic carriers of HBV (Hollinger, 1985).

Chronic hepatitis B caused by HBV infection is identified by a positive blood test for hepatitis B virus surface antigen (HBsAg). Chronic persistent hepatitis is not usually considered to be progressive, and the liver function

of such patients continues to be normal or near normal. Some chronically infected patients, however, may progress to chronic active hepatitis which is defined by the presence of elevated liver enzymes [Alanine aminotransferase (ALT) or Aspartate aminotransferase (AST)] and characteristic features noted on the histological examination of a liver biopsy. Chronic active hepatitis may result in cirrhosis which in some cases, leads to death. The carrier state, whether associated with chronic liver diseases or not, may lead to primary hepatocellular carcinomas (PHC), which is one of the most prevalent carcinomas in man (Di Bisceglie *et al.*, 1988, Seeff and Koff, 1986).

The concept that the chronic carrier state is the leading cause of the development of PHC has been supported by three lines of evidence. Firstly, the geographic distribution and the incidence of HBV infection and PHC have been found to coincide. For example, in 1978, Szmuness (1978) reported that regions in which HBV infection was very highly prevalent also showed high incidences of PHC, observations that suggested a causal relationship between HBV infection and the development of PHC. The second piece of evidence that HBV infection was related to PHC came from the high rate of HBV infection among the patients with hepatocellular carcinoma. Several studies conducted in Korea (Chung *et al.*, 1983, Sjogren *et al.*, 1984) indicated that 85%-95% of patients with hepatocellular carcinoma had current

or past infections with HBV, suggesting the possible causal role of HBV in the development of PHC. The most convincing evidence for the linkage of HBV infection with PHC came from a prospective study of 22,707 Taiwanese males (Beasley *et al.*, 1981). The HBsAg carriers in this study were found to develop PHC at a significantly higher rate than the non HBsAg carriers. The results presented by Beasley and coworkers indicate that the relative risk of developing hepatoma is approximately 200 times higher for carriers compared with non-carriers. Further studies of correlation between HBV infection and development of PHC have substantiated the conclusion that HBV plays a primary role in the etiology of PHC (Di Bisceglie *et al.*, 1988, Maupas and Melnick, 1981).

1.1.2 Epidemiology of HBV Infection.

Chronic HBV carriers are found worldwide, with an estimated 300 million chronic carriers. An extensive global survey demonstrated a remarkable pattern to the geographic distribution of HBsAg carriers. Chronic HBV infection was very common in China, Southeast Asia, and sub-Saharan Africa, where 5%-20% of the population was found to be HBsAg positive. It was less frequent in Central and South America, the Middle East, the Mediterranean area, Southern and Eastern Europe, Russia and India where 1%-5% of the population tested HBsAg positive. The incidence of chronic HBV infection was found to be rare in the countries of Western Europe, North America, Australia and New Zealand, with only 0.1%-1% of the

population testing positive for HBsAg (Maupas and Melnick, 1981, Hollinger and Melnick, 1985). In addition, other studies have shown that chronic HBV infection was common among high risk groups such as intravenous drug abusers, transfusion recipients, male homosexuals, medical care personnel and persons of Chinese and Southeast Asian origins (Hoofnagle and Alter, 1984). This review also indicated that in the general US population only 0.2% were HBsAg carriers, while the incidence was 6% in homosexuals, 7% in drug addicts and hemophiliacs, and 10% in Asian-Americans.

1.1.3 Transmission of HBV.

The mode of HBV transmission can be divided into two categories: vertical transmission and horizontal transmission (Hollinger and Melnick 1985). Vertical transmission is defined as the transmission of HBV from an HBV infected mother to her offspring. Although most of these infants acquire the infection at birth, a small percentage (<5%) may acquire the infection *in utero* (Liu *et al.*, 1990). There are several important observations related to vertical transmission. The ethnic origin of the mother seems to be a factor. Vertical transmission in patients of Chinese origin is common. However, despite the high incidence of HBsAg carriers in sub-Saharan Africa, vertical transmission is less frequent. In this geographical area the disease is acquired in early childhood, but the mechanism of transmission remains unclear (Ayoola, 1988). Another important factor affecting

vertical transmission is the antigenic status of the mother. Several investigators (Beasley *et al.*, 1977, Okada *et al.*, 1976, Skinhoj, *et al.*, 1976) reported that infants have a much higher chance of becoming chronic carriers if the mother is HBeAg positive. In contrast anti-eAg positive mothers, despite being HBsAg positive, rarely transmit HBV infection to their infants. This indicated that the presence of infectious virus particles in the sera of mothers would result in vertical transmission, since HBeAg positive patients are most likely to be HBV DNA positive in sera as well (Sallberg, *et al.* 1988). The large number of HBsAg carriers throughout the world provides an important reservoir for vertical transmission which has, in turn, been considered the most important mechanism for the maintenance of high HBV carrier rates in certain geographic areas, such as in Southeast Asia.

Horizontal transmission of HBV can take place by percutaneous transmission, through sexual contact or close household contact. Percutaneous transmission occurs when blood transfusion recipients receive blood from HBV positive blood donors or by direct transfer of the virus among intravenous drug abusers who share contaminated needles or syringes. It may also occur among persons living in areas where medical care is poor and decontamination of medical tools may not be complete (Hollinger and Melnick, 1985, Ayoola, 1988). Sexual transmission is not surprising since

the virus has been identified in semen and cervical secretions (Ganem, 1982, Yoffe, *et al.* 1990). Close household contact between infected and non-infected individuals is another mode for the horizontal transfer of HBV. The infection may be transmitted via contact between family members as demonstrated, for example, by the study of HBV infection in Italy families (Mazzotta *et al*, 1990).

The development of an effective vaccine for HBV has decreased the incidence of this disease in some high risk groups. However, the cost of the vaccine has precluded its widespread use even in most industrialized countries. Several countries now advocate HBsAg screening during pregnancy and routine vaccination of newborn infants to break the cycle of vertical transmission. This practice may eventually decrease the incidence of HBsAg carriers.

1.2 The Hepadnaviridae Family.

1.2.1 History of Hepatitis B Virus.

HBV was identified as the etiological agent in viral hepatitis by Blumberg and his colleagues (Blumberg *et al.*, 1965). The sequence of events began in 1965 with the discovery of an unidentified antigen from the serum of an Australian aborigine. This antigen was called the Australian (Au) antigen, and was subsequently detected with high

frequency in the sera of patients with leukemia, Down's syndrome and hepatitis (Blumberg *et al.*, 1965). Further research revealed that the antigen was transmissible through sera and was associated with acute viral hepatitis. Thus the etiological role of "Au" antigen in "serum" hepatitis was established (Blumberg *et al.*, 1967, Blumberg, 1977).

In 1970, Dane and Cameron (1970) purified virus particles from the sera of Au antigen positive hepatitis patients. Electron microscopy of the purified product identified three distinct particle forms: a spherical particle approximately 42 nm in diameter, a spherical particle of 22 nm, and a 22-nm filamentous particle. The 42-nm spherical particle is composed of an outer shell and an inner nucleocapsid associated with the viral genome. This particle, referred to as the Dane particle, is the complete infectious form of the virus. The other two particles, both 22 nm in diameter and having either spherical or filamentous morphology, are not infectious. These latter two particles are found in abundance in the sera of infected individuals. The Australian antigen was identified as a component of the viral envelope and is now called hepatitis B surface antigen.

1.2.2 Genetics of HBV.

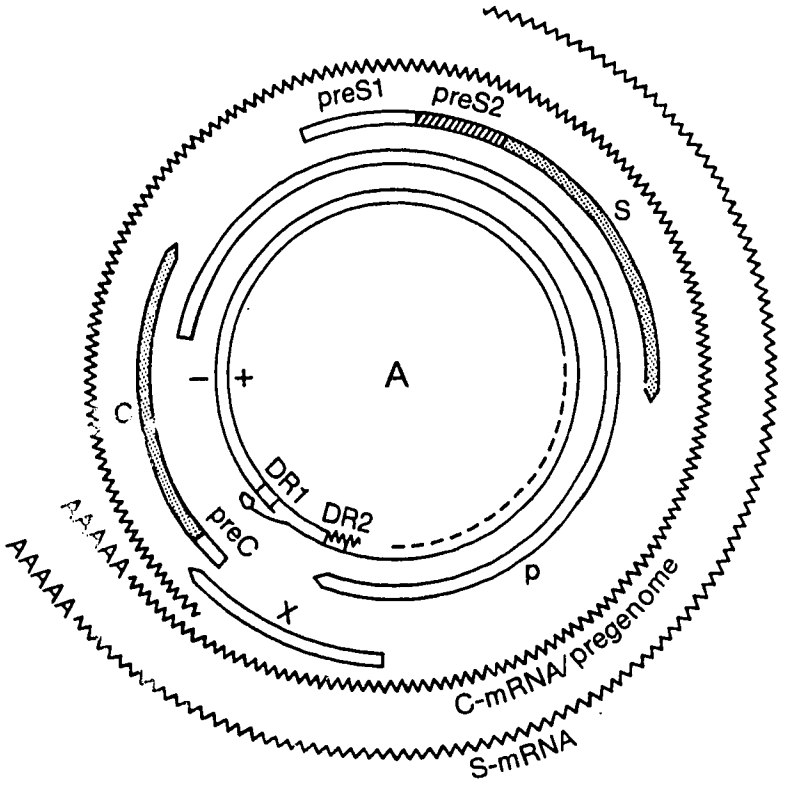
Characterization of HBV has revealed it to be different from other mammalian viruses (Blum *et al.*, 1989, Ganem and Varmus, 1987). The genome of the HBV virion consists of a

small circular DNA molecule existing as a partially double stranded structure with a single stranded region of variable length (figure 1.1 A, Robinson *et al.* 1974, Summers *et al.*, 1975, Landers *et al.*,1977). Of the two DNA strands, the full-length strand is complementary to viral mRNA and is termed the minus strand, while the incomplete DNA molecule possesses the same polarity as viral mRNA and is therefore designated as the plus strand. The minus strand has a 9 nucleotide terminal redundant sequence, thus it is a little longer than one unit length (Lien, *et al.*, 1987). A protein is covalently attached to the 5' end of the minus strand (Gerlich and Robinson, 1980). The incomplete strand contains a nucleotide sequence of only 50%-80% of the length of the minus strand. The 5' end of the plus strand is linked to an oligoribonucleotide and is located at the region of 50-300 nucleotides downstream from the 5' end of minus strand (Lien *et al.*, 1986, Will *et al.*, 1987). Through this cohesive overlap between the two strands, the circular structure of the two linear forms of viral DNA is maintained. The circular structure of partially double stranded DNA thus consists of a covalently bound protein at the 5' end of the minus strand DNA which has a short redundant sequence and a gap in the plus strand (Fig. 1.1. A, Summers *et al.*, 1975, Lander *et al.*, 1977).

Viral DNA polymerase activity has been found associated with the Dane particle preparation. This can incorporate

Figure 1.1.

Structure and genetic organization of mammalian hepadnaviruses (A) and DHBV (B). The two inner circles represent the two strands of genomic DNA and the dashed line shows the varying length of the plus strand DNA in mammalian hepadnaviruses. The plus strand of the genome in DHBV is almost complete. Two direct repeat regions are indicated by DR1 and DR2. A protein primer at the 5' end of the minus strand is indicated by the symbol (⟳) and the RNA primer of the plus strand is indicated by (∿). The open arrows represent the ORFs with different initiation codons in each ORF. The outer wavy lines represent the major RNA transcripts produced by the hepadnaviruses.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DR1
DR2
preC
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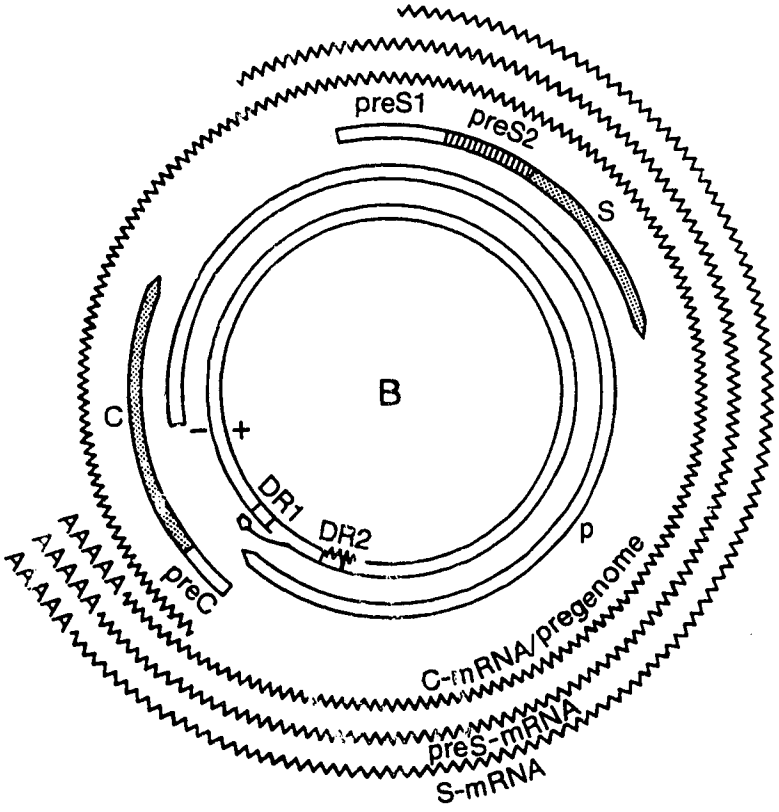
AAAAA
C-mRNA/pregenome
S-mRNA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DR2
preC
p
AAAAA
C-mRNA/pregenome
preS-mRNA
S-mRNA

radiolabelled deoxynucleotides into DNA *in vitro* as detected by acid precipitation (Kaplan *et al.*,1973). This enzymatic activity has been identified in virion preparations (Dane particles) as well as in virus core particles which were obtained from Nonidet P-40 treated virions (Robinson and Greenman, 1974). The radiolabelled deoxynucleotides were found to be principally incorporated into the plus strand near the 3' end, indicating that the DNA polymerase of the virus can repair the single strand gap to make the virus genome a fully double stranded molecule (Summers *et al.*, 1975, Landers *et al.*, 1977, Hruska *et al.*, 1977).

1.2.3 Hepatitis B-Like Viruses in Animals.

The DNA polymerase activity associated with HBV in the serum provides a convenient method for the detection of similar hepatitis B viruses in other species. The first HBV related virus identified was found in a colony of woodchucks (*Marmota monax*) in the Philadelphia Zoo (Summers *et al.* 1978). It had been noted that the most frequent cause of death in these animals was hepatocellular carcinoma associated with preexisting chronic active hepatitis. A viral etiology of the hepatitis and hepatoma in these animals was suggested by Robert Snyder, a veterinarian at the Philadelphia Zoo. Following his suggestion, Summers *et al.* (1978) demonstrated DNA polymerase activity in serum samples from woodchucks which suggested the presence of a HBV-like virus in these animals. Further studies confirmed the

existence of a virus with a genomic structure and serological markers similar to those of HBV. The antigen of the virus core particles was able to react with antisera prepared for HBV indicating antigenic cross reactivity between these two viruses (Summers *et al.*, 1978, Werner *et al.*,1979). Another animal hepadnavirus was found in the Beechey ground squirrels (*Spermophilus beecheyi*) living in a region of northern California (Marion *et al.*, 1980). Characterization of this virus revealed similarities in ultrastructure, genomic structure and enzymatic activity to HBV (Marion *et al.*, 1980, Ganem *et al.*, 1982). An avian hepatitis virus constitutes the fourth member of the hepadnavirus family and was first detected in domestic Pekin ducks from the People's Republic of China (Summers, 1981). In the process of attempting to pass this avian virus to Pekin ducks in North America, Mason *et al.* (1980) found that some of the Pekin ducks commercially purchased in the USA were already infected with a similar virus. Although the avian hepatitis virus is more divergent from HBV than are the other animal hepatitis viruses, the avian hepatitis virus has many characteristics in common with all members of the hepadnavirus family. There have been reports of hepatitis viruses in other animals, however, woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV) are the best characterized animal hepadnaviruses (Marion, 1988).

1.2.4 Properties of Hepadnaviruses.

The characteristics of hepatitis B viruses have been summarized by Summers (1981) and Marion (1988). The viruses in the three mammalian species are quite similar to each other, while the avian virus diverges somewhat from the other members of the family. The virions of HBV, WHV, GSHV and DHBV are all spherical in form with sizes ranging from 40 to 47 nm. Viral particles from HBV, WHV and GSHV exhibit CsCl buoyant densities in the range of 1.225-1.24 g/ml, however, DHBV particles have a relatively low buoyant density of 1.16 g/ml. The sera from hepatitis B infected mammalian hosts contains an excess of both spherical and filamentous forms of surface antigen particles, whereas in DHBV infected animals, the sera contains only large, convoluted spherical particles in addition to infectious particles. The core antigen (cAg) and surface antigens (sAg) of WHV and GSHV can cross react with those of HBV. No cross reactivity of cAg and sAg has been found between DHBV and HBV.

The genome size of the viruses has been identified as 3,188 base pairs (bp) for HBV, 3,308-3,320 bp for WHV, 3,311 bp for GSHV and 3,021 bp for DHBV. Although the single stranded gap in the genome varies, it is generally small in the DHBV genome, i.e. the DHBV genome has a high percentage of double stranded DNA (Lien *et al.*, 1987). In the case of mammalian viruses, the double stranded region varies from 50% to 80% of the genome. The nucleotide sequence homology between the genome of HBV and the animal viruses is about 70%

for WHV, 55% for GSHV and 40% for DHBV (Tiollais *et al.*, 1985).

Hepatitis B viruses are active in a narrow host range. HBV can infect man and some higher primates, such as chimpanzees; WHV only infects woodchucks; GSHV can infect both ground squirrels and woodchucks; DHBV has been reported to be infectious in ducks and geese (Marion 1988). Research on the hepadnaviruses has been hampered by the inability of these viruses to infect conventional laboratory animals. Infection with hepatitis B virus is hepatotropic, although viral DNA has been detected in other organs, such as pancreases, lymphocytes and spleen. The majority of virus replication however occurs in hepatocytes.

Based on their distinct properties, HBV, WHV, GSHV and DHBV have been classified into a family designated hepadnaviridae (Gust, 1986) and the hepatitis-B-like viruses thus are called hepadnaviruses.

1.3 Molecular Biology of Hepadnaviruses.

1.3.1 Genomic Organization of the Viruses.

Hepadnavirus DNA extracted from virions has been cloned and the complete nucleotide sequences of HBV, WHV, GSHV and DHBV have been established (Galibert *et al.*, 1979, Galibert *et al.*, 1982, Kodama *et al.*, 1985, Seeger *et al.*, 1984,

Mandant *et al.*, 1984, Sprengel *et al.*, 1985). Analysis of three reading frames of the nucleotide sequences has revealed that, in all four viruses, the open reading frames (ORF) for gene expression reside only on the minus strand DNA. There are four ORFs found in the mammalian hepadnavirus genomes, and three ORFs in the DHBV genome as shown in figure 1.1.

The key feature to be noted in figure 1.1 is the compact organization of hepadnavirus genomes (Miller *et al.*, 1989). The coding sequences for protein products are found in all three possible reading frames and the ORF for the P gene overlaps each of the other viral protein ORFs. The amino acid sequence of each ORF is unique by utilization of alternative reading frames although one ORF overlaps with the other. The ORF encoding the surface antigens (ORF S) is relatively complex and consists of two parts. The S gene is located in the 3' portion of the coding region. Upstream of the S gene is a region designated pre S. There are two in-phase frames in the pre S region: pre S2 immediately precedes S gene and pre S1 precedes pre S2. Nucleocapsid polypeptides are encoded by ORF C. Similar to the surface antigen gene structure, ORF C contains the C gene and pre C regions. The longest open reading frame, ORF P, is presumed to code for the viral polymerase. This ORF encompasses the whole ORF S and overlaps with ORF C and, in the mammalian viruses, ORF X. The shortest open reading frame is ORF X which is present only in the mammalian hepadnaviruses.

1.3.2 Gene Products of Hepadnaviruses.

Hepadnaviruses surface antigen particles isolated from the sera of hepatitis B virus infected individuals have been analyzed by SDS-polyacrylamide gel electrophoresis. These analyses have revealed that various sizes of surface antigen polypeptides are present on the virus envelope (Feitelson *et al.*, 1983). The major surface antigen (sAg) encoded by gene S ranges from a molecular weight of 17 kDa in DHBV, to 22 kDa in WHV and GSHV, and 24 kDa in HBV (Schlichit *et al.*, 1987a, Marion *et al.*, 1983, Feitelson *et al.*, 1983). Additional polypeptides with molecular weights greater than the major surface antigen are found in all hepadnaviruses, but are present in smaller amounts than the major surface antigen. The larger molecular weight gene products (pre sAg) are encoded by the ORF including pre S and S regions (Feitelson *et al.*, 1983, Schlicht *et al.*, 1987). Glycosylated versions of these surface proteins, namely S, pre S_2 S, and pre S_1 S_2 S, have been detected in HBV, WHV and GSHV, but there is no evidence for the presence of glycosylated forms of DHBV surface proteins (Schlichit *et al.*, 1987, Pugh *et al.*, 1987). Studies on the structure and function of hepatitis B surface antigens suggest that sAg is a structural protein that has little or no immunogenicity, whereas the preS proteins with N-terminals exposed on the surface of the virion are more immunogenic (Heermann *et al.*, 1984, Tiollais *et al.*, 1985, Neurath *et al.*, 1985, Schlichit *et al.*, 1987). A hepatocyte

binding activity has been speculated for HBV pre S2 protein, because the pre S2 encoded sequence contains a receptor for polymerized human serum albumin (pHSA) and the same receptor has been found in hepatocytes. It is thus postulated that pHSA mediates the attachment of HBV to hepatocytes (Tiollais, *et al.*, 1985). A similar binding activity has been suggested for the pre S1 protein as well (Neurath, *et al.*, 1986). However, using recombinant hepatitis B virus surface antigen as a probe to test the cell receptor in various cell lines, Peeples, *et al.* (1987) have demonstrated that small sAg alone can act as the viral attachment protein, suggesting the possible role of sAg in viral attachment to host cells. However, such a function has not been demonstrated in WHV (Pohl *et al.*, 1986). In addition, a recent report by Summers *et al.* (1990) provides evidence that viral surface antigen plays a role in regulation of viral covalently closed circular DNA (cccDNA) pools and in the establishment of persistent infections.

Core antigen (cAg), the principal structural protein of the viral nucleocapsid, is encoded by the C gene. The amino acid composition of cAg has been determined from the nucleotide sequence analysis of the C gene and reveals a conserved, highly basic carboxy terminal, which may be related to the nucleic acid binding function of this protein (Mandart *et al.*, 1984, Petit and Pillot, 1985). Phosphorylated forms of cAg have been found in core particles

isolated from the cytoplasm of infected hepatocytes, but not in the core particles obtained from virions, suggesting a possible link between dephosphorylation and virion maturation (Roossinck and Siddiqui, 1987, Pugh *et al.*, 1987). The synthesis of pre C polypeptide has been found to be independent from the synthesis of cAg since mutations in the pre C region do not affect the production of cAg or virus formation (Jean-Jean *et al.*, 1989, Schlicht *et al.*, 1987b). The expression of pre C, however, is required for secretion of eAg, a processed peptide from the ORF of C which is found in circulating blood (Roossinch *et al.*, 1986, Schlicht *et al.*, 1987b).

It was expected that the longest ORF of hepadnavirus would code for the viral polymerase, an enzyme associated with the virion or core particles (Kaplan *et al.*, 1973). In addition, it has been suggested that the viral polymerase bears multiple functions (Summers and Mason, 1982, Robinson *et al.*, 1987, Khudyakov and Markhov, 1989). Recently, several experiments have provided convincing evidence to support the prediction that the longest ORF encodes for the viral polymerase. This ORF would code for a protein of approximately 90 kDa. Employing a technique of direct detection within the gel of enzymatic activity of proteins following SDS-PAGE electrophoresis, Bavand and Laub (1988) were able to demonstrate DNA polymerase (DNA pol) and reverse transcriptase (RTase) activities associated with HBV proteins

secreted by a transfected hepatoma cell line. The protein bands found to contain these two polymerase activities were located at 70 kDa for the major activity and at 90 kDa for a minor activity. The protein in the major band reacted with antisera directed against the carboxy terminus of the ORF P gene product, suggesting that this 70 kDa peptide is derived from ORF P (Bavand *et al.*, 1989). During the study of the enzymatic activity of DHBV-associated polymerase, a group of investigators (Bosch *et al.*, 1988, Radziwill *et al.*, 1988, Bartenschlagen and Schaller, 1988) have found that a DHBV P gene product is tightly bound to the 5´end of the viral minus strand DNA. This has led to the belief that the terminal protein is encoded by the N-terminal domain of P gene. Furthermore, Radziwill *et al.* (1990) have contributed to our understanding of HBV biology by constructing a series of the P gene mutants of HBV and assaying the subsequent gene products for biological activity by transient expression in tissue culture. In addition to locating the polymerase and terminal protein domains, they have identified an RNase H active domain residing near the C-terminus of the P gene. They have noted that the mutants of the P gene can be complemented *in trans* by wild type P gene product, but not by a second P gene mutant. This indicates that the P gene functions as a single translational unit. The functional domains on the P gene are arranged in the following order: terminal protein, spacer, RTase/DNA pol and RNase H. Recently, Noonan *et al.*(1990) have cloned the intact P gene

into a baculovirus system. The P gene product expressed in insect cells was found to be a 90 kDa protein and was able to react with the antiserum directed against a synthetic peptide representing the C-terminal sequence of the P gene. However, enzymatic activity of the expressed P gene product has not been detected as yet.

The smallest ORF of hepadnaviruses, designated X, is only found in the mammalian viruses. In HBV this ORF has the capacity to encode for a protein of approximately 17 kDa in size. The gene of ORF X has been expressed *in vitro* using recombinant DNA. This X gene product has been identified by immunoprecipitation with specific antisera produced against X gene peptides as well as with sera from HBV infected individuals (Pfaff *et al.*, 1987, Siddiqui *et al.*, 1987 Koike *et al.*, 1988,). The function of the X gene has not been completely determined. Recent studies by Wu *et al* (1990) suggest that the X gene product has some protein kinase activity. It also has been demonstrated that the X gene product can transactivate HBV and sequences of some other viruses (Spandau and Lee, 1988, Twu and Robinson, 1989). The X gene is speculated to have a possible role in the development of hepatocellular carcinoma. This is implied from the fact that some mammalian viruses contain the X gene and induce hepatocellular carcinomas in their host animals. The duck virus lacks an X gene and persistent infection with DHBV rarely results in PHC. In an effort to define the role

of X gene in the etiology of PHC, studies with transgenic mice have produced variable results. Earlier studies failed to show a high incidence of tumors in transgenic mice (expressing the X gene), however, a recent study by Kim *et al.* (1990) has shown that two of three strains of transgenic mice developed hepatocellular adenomas and hepatocellular carcinomas at ages of 9 and 11 months, respectively. This indicates that this product of the virus may be one of the elements involved in development of hepatocellular carcinoma. However, tumors have been produced in mice transgenic for the HBsAg. There animals show considerable inflammation of the liver and inflammation alone may be a significant factor (Chisari, *et al.*, 1989).

1.3.3 Gene Transcripts and Signal Sequences for Gene Expression.

Two classes of RNA transcripts are present in major amounts in hepadnavirus infected livers: genomic and subgenomic transcripts (figure 1.1) (Cattaneo and Schaller 1984, Moroy *et al.*, 1985, Enders *et al.*, 1985, Buscher *et al.*, 1985). The largest genomic RNA transcripts are initiated in or upstream of the pre C region and ended inside the C gene. They encompass the entire genome and are slightly redundant (approximately 200 nucleotides). The other major transcripts are subgenomic and contain one major species and two minor species in the case of mammalian viruses or two major species of transcripts in the avian

virus. The 5' end of the subgenomic RNA transcripts begins upstream to pre S, or near pre S2 and terminates at the same site as pregenomic RNA in the C gene. The hepadnavirus RNA transcripts are polyadenylated, have plus-stranded polarity and were thought to be unspliced. However, it has recently been reported that spliced transcripts are found in HBV infected cells (Su *et al.*,1989, Chen *et al.*, 1989, Suzuki *et al.*, 1989). The function of these spliced transcripts remains to be clarified.

The biological function of each major transcript is suggested from the ORFs covered by each transcript. Genomic RNA transcripts are thought to act as mRNA for core antigen or e-antigen and polymerase gene expression and serve as templates for reverse transcription. The internally located initiation site of ORF P on the genomic RNA transcript raises the question of how viral polymerase production occurs. Two independent groups (Chang *et al.*, 1989, Schlicht *et al.*, 1989) have reported *de novo* translation of the polymerase with initiation occurring at a unique P gene AUG, rather than as a result of a frame shift from gene C . Furthermore, the reported existence of spliced transcripts in HBV infected cells suggests a possible role of the spliced RNA species in polymerase translation (Chen *et al.*, 1989). Subgenomic transcripts are principally responsible for surface antigen expression.

There are two direct repeats located in the cohesive ends of the viral DNA genome. Each direct repeat is about 11 nucleotides long and they are separated by a spacer of 50-200 nucleotides. In all hepadnaviruses the two direct repeats are highly conserved and play an important role during viral replication (Miller *et al.*, 1989).

There are a few types of sequences that appear to be involved in control of hepadnavirus gene expression. Although the virus relies on cellular RNA polymerase II to perform the steps of transcription (Ganem and Varmus, 1987), there is a "TATA" box like sequence found at or near the positions predicted for RNA polymerase binding (Miller, *et al.* 1989). This is interesting because we do not know how cellular RNA polymerase recognizes the transcriptional signal. However, the viral genome contains conserved regions at or near the predicted positions for promoters. Thus it has been suggested that the promoter elements may be unique in hepadnaviruses (Miller *et al.*, 1989). Only one polyadenylation signal has been located in the entire genome and this signal resides within the C gene region. From analysis of the 3' ends of hepadnavirus transcripts, this polyadenylation signal seems to be responsible for polyadenylation of all the viral transcripts. There are two enhancer elements and a glucocorticoid-responsive element present in the hepadnavirus genome, and they may be involved in the regulation of viral gene expression (Bulla and

Siddiqui, 1988, Miller *et al.*, 1989 Lopez-Cabrera, *et al.*,1990).

1.4 Replication of Hepadnaviruses.

Elucidation of the DHBV replication mechanism by Summers and Mason (1982) is a landmark in the molecular biology of hepadnaviruses. The observation that DHBV infected duck liver contains a large amount of free minus strand DNA lead these investigators to attempt to isolate the viral replicative complex and to study minus strand DNA synthesis. The isolated replicative complexes were found to be capable of both plus and minus strand DNA synthesis when assayed by endogenous labelling. This is in contrast to the mature virion which mostly repairs the single stranded gap in plus strand DNA. Further analysis of endogenously labelled products from the replicative complexes showed that the synthesis of minus strand DNA was resistant to actinomycin D, which inhibits DNA-directed DNA synthesis but not RNA-directed DNA synthesis. In addition, the minus strand DNA was sensitive to S1 nuclease, an enzyme that selectively degrades single stranded polynucleotides. When the extracted nucleic acids from cores were tested as templates for avian myeloblastosis virus (AMV) DNA polymerase, these templates were sensitive to the treatment with ribonuclease (RNase), indicating that an RNA-directed DNA synthesis was involved.

Since the newly synthesized minus strand DNA product is mainly single stranded in form, as defined by sensitivity to S1 nuclease, the RNA template must be degraded as the minus strand is synthesized. Synthesis of plus strand DNA, on the other hand, is sensitive to actinomycin D but more resistant to S1 nuclease digestion than is minus strand DNA, suggesting that plus strand DNA is synthesized from a DNA template and remains bound to the template. These observations led Summers and Mason to propose a general model for replication of the genome of hepatitis B-like viruses. It has similarities to the replication of retroviruses in which the replication flows from genomic RNA to DNA intermediates and back to genomic RNA. In the hepadnavirus the information flow is from genomic DNA to an RNA intermediate and back to genomic DNA.

The hepadnavirus replication process has been studied in detail for DHBV (Summers and Mason, 1982, Mason *et al.*, 1982, Molnar-Kimber *et al.*, 1984, Lien *et al.*, 1986), for GSHV by Seeger *et al.* (1985) and for HBV from both chimpanzee and human sources by Will *et al.* (1987). The pregenomic RNA, which is used as the template for the minus strand DNA synthesis, is heterogeneous at its 5' end. It has been suggested that the shortest species are most likely utilized in viral replication (Enders, *et al.* 1987, Seeger *et al.*, 1985, Will *et al.*, 1987). Synthesis of minus strand DNA is initiated within DR1 and primed by a protein (Gerlich and

Robinson, 1980, Molnar-Kimber *et al.*, 1983, Lien *et al.*, 1987). The complete minus strand DNA possesses a short terminal redundancy. Synthesis of plus strand DNA is initiated adjacent to the DR2 sequence and primed with a 5' end capped oligoribonucleotide. The sequence of this oligonucleotide was found to be identical to the 5' end region of pregenomic RNA, suggesting that the primer originates from the degraded pregenomic RNA template (Lien *et al.*, 1986).

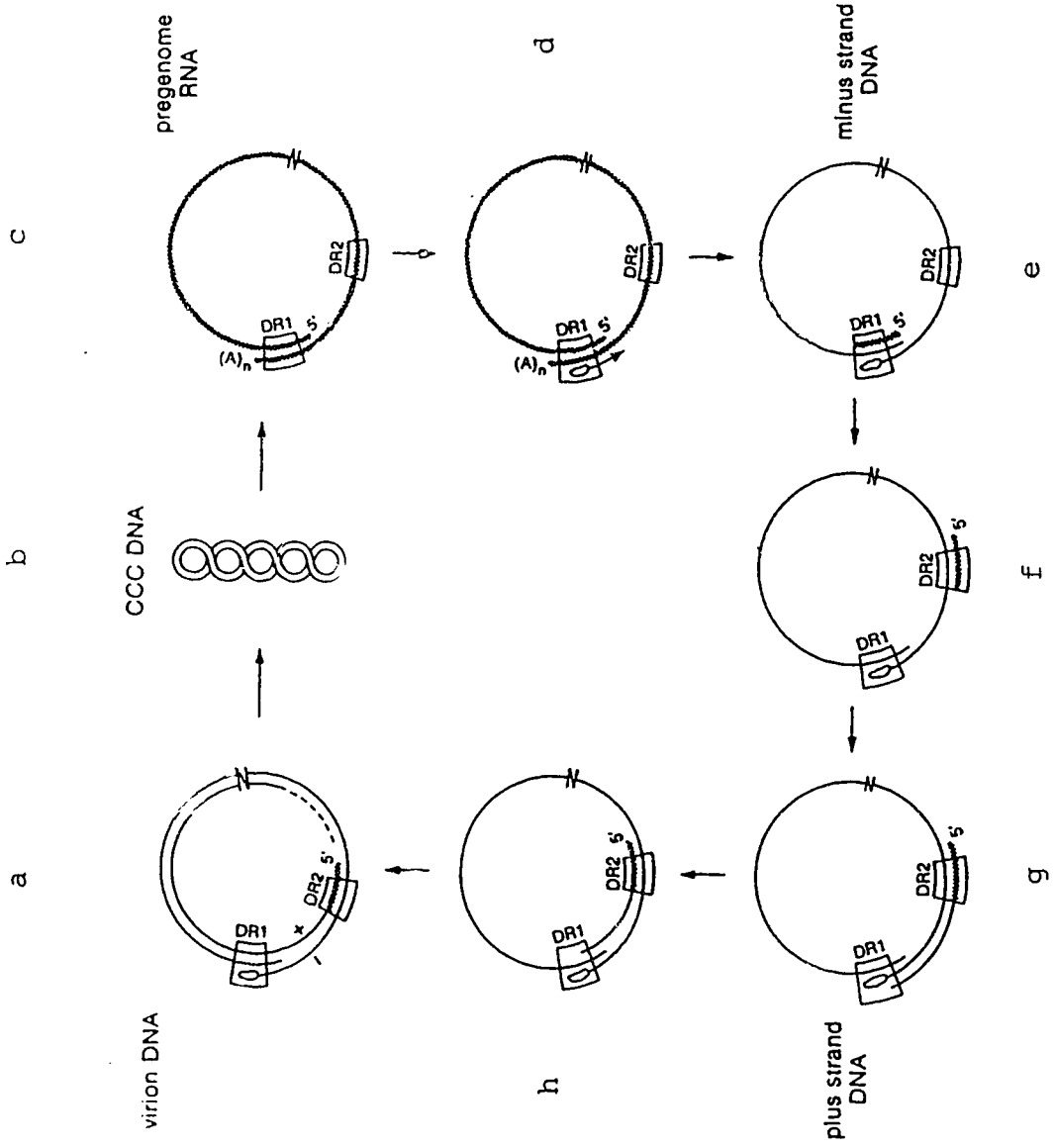
The model for hepadnavirus replication shown in figure 1.2 is a composite of the initial replication pathway proposed by Summers and Mason (1982) and subsequent details contributed by Lien *et al.* (1986), Tuttleman *et al.* (1986a), Seeger *et al.* (1986) and Will *et al.* (1987). The complete replicative process can be divided into four major steps:

i. Conversion of genomic DNA (RC) into cccDNA.

The earliest event in viral replication following liver cell infection is the generation of covalently closed circular DNA (cccDNA) in the cell nucleus (Mason *et al.*, 1983) (Fig. 1.2 a and b). Detectable levels of this cccDNA appear as early as 6 hours post-infection, preceding the synthesis of the major RNA transcript and the reverse transcription step (Tagawa *et al.*, 1986). Using BUdR labelling of intracellular DHBV DNA and isopynic CsCl density gradient centrifugation to isolate total cccDNA, Tuttleman *et*

Figure 1.2.

The replication pathway of the hepadnaviruses. Viral DNA strands are shown by the solid lines and RNA is represented with wavy lines. Two direct repeat regions are shown by open boxes marked DR1 and DR2. The symbol (⊂⊃) in the DR1 box represents the terminal protein.


a
b
c
d
e
f
g
h
virion DNA
CCC DNA
pregenome RNA
minus strand DNA
plus strand DNA
DR1
DR2
5'
(A)n

al. (1986a) have shown that cccDNA is amplified from newly synthesized relaxed circular DNA produced in the cytoplasm through *de novo* synthesis, rather than from semiconservative replication of preexisting cccDNA in the nucleus. Conversion of the partially double stranded genome into cccDNA requires removal of both the short redundant sequence with the primer protein at the 5′ end of minus strand DNA and the oligoribonucleotide at the 5′ end of plus strand.DNA. This results in a complete minus strand and gap in the plus strand which must be filled before the ends of both strands can be ligated into circles.

The pool of cccDNA located in the cell nucleus is maintained at a certain size which seems to be regulated to a level that is sufficient for virus production but below the cytotoxic level for the host cell. This regulation of viral pool size may be very important in the establishment of persistent infection (Tuttleman *et al.*, 1986a). Recent work reported by Summers *et al.* (1990) has shown that envelope proteins have a negative regulatory effect on the amplification of cccDNA. The principal function of cccDNA is to serve as template for production of mRNA and pregenomic RNA.

ii. Synthesis and encapsidation of pregenomic RNA.

Pregenomic RNA is transcribed from the cccDNA template in the nucleus and it is assumed that host RNA polymerase is

involved in this transcription (Ganem and Varmus, 1987). Transcription is initiated upstream of the polyadenylation site and near or within the pre C region of the genome. Termination of transcription occurs at the original polyadenylation site after completion of the complete genomic transcript. Thus the poly(A) addition site is utilized upon first passage, but is utilized on the second passage. As a result, the transcription product is longer than the genome and contains a terminal redundancy with two DR1 sequences, one located at the 5' end and the other near the poly(A) tail at the 3' end, and one DR2 sequence adjacent to the DR1 region at the 3' end (Fig.1.1 and Fig. 1.2 c).

The polyadenylated pregenomic RNA is transported to the cytoplasm and encapsidated into the nucleocapsid of the virus. By expressing the C gene products in *E. coli*, Birnbaum and Nassal (1990) have found that both the RNA transcript encapsidation into core particles and the particle stability are dependent on expression of the arginine rich region located near the carboxy terminal end of the core protein although the entire arginine rich domain of the core protein is not necessary for assembly. It is known that the 5' end of pregenomic RNA is heterogeneous, but which RNA transcripts are preferred for encapsidation remains an interesting question. Enders *et al.* (1987) have examined GSHV pregenomic RNA packaging and have shown that only the shortest of the genomic RNA species is found in the core

particles. It is known that at least pregenomic RNA, viral polymerase and the terminal protein which originates from the N-terminal portion of the polymerase are assembled with the nucleocapsid protein (C gene product) into a core particle.

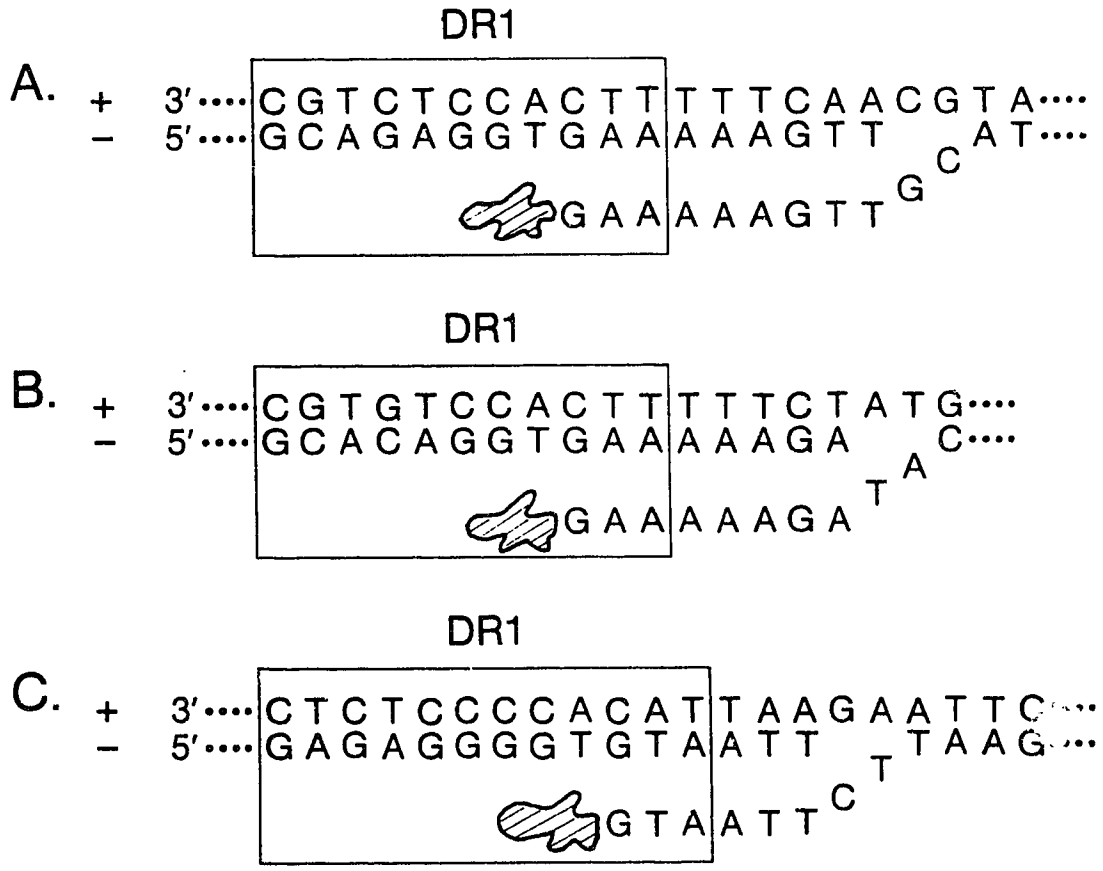
iii. Synthesis of the minus strand of DNA.

Viral minus strand DNA is synthesized by reverse transcriptase within core particles in the cytoplasm. By S1 nuclease mapping and 5' primer extension, several investigators (Molner-Kimker *et al.*, 1983, Seeger *et al.*, 1986, Will *et al.*, 1987) have identified the initiation site of minus strand DNA synthesis within the DR1 region, beginning with the third residue from the 3' end of DR1 (Fig. 1.2 d). As shown in figure 1.3, the sequence linked to the terminal protein begins with deoxyguanosine for all the hepadnaviruses. The sequences are Protein-d(GAA...) for HBV, Protein-d(GAA...) for GSHV and protein-d(GTA...) for DHBV.

It is interesting to note that the first nucleotide linked to the terminal protein is conserved in all hepadnavirus sequences. This may be related to recognition of deoxyguanosine by viral polymerase, or more specifically by the terminal protein domain of the polymerase, for the initiation of the minus strand DNA synthesis. By genetic analysis of the origin of minus strand DNA synthesis in WHV, Seeger *et al.* (1990) have shown that the terminal protein can be linked to dG or dA, but not to any pyrimidine

Figure 1.3.

. Nucleotide sequences at the 5´ end of the minus strand DNA of HBV (A), GSHV (B) and DHBV (C). The DR1 region is contained in the square. The shaded structure represents the terminal protein.


DR1
A.
+ 3′···· CGTCTCCACTT TTTCAACGTA····
− 5′···· GCAGAGGTGAA AAAGTT AT····
C
G
GAA AAAGTT
DR1
B.
+ 3′···· CGTGTCCACTT TTTCTATG····
− 5′···· GCACAGGTGAA AAAGA C····
A
T
GAA AAAGA
DR1
C.
+ 3′···· CTCTCCCCACAT TAAGAATTC····
− 5′···· GAGAGGGGTGTA ATT TAAG····
T
C
GTA ATT

deoxynucleotide. Their results suggest a preference for purine deoxynucleotides for recognition by terminal protein.

There are two DR1 regions available for initiation of minus strand DNA synthesis (Fig.1.2 d). It is assumed that the virus uses the DR1 region at the 3' end of pregenomic RNA for initiation since the synthesis can be completed without interruption (Ganem and Varmus, 1987) and the 5' end of the pregenomic RNA coincides exactly with the 3' end of the minus strand DNA. The RNA template is degraded by RNase H as the synthesis of minus strand DNA proceeds. The 3' end of minus strand DNA passes through the DR1 region at the 5' end of RNA transcript and shortly thereafter synthesis is terminated. Therefore, the minus strand DNA also has a short terminal redundant sequence which may be important for the synthesis of the second DNA strand.

iv. Synthesis of the plus strand of DNA.

Sequence data of DHBV have shown that an oligoribonucleotide linked to the 5' end of plus strand DNA is identical to the sequence of the 5' end of pregenomic RNA, suggesting that this oligoribonucleotide primer may be derived from the pregenomic RNA template. The physical mapping of the DHBV genome has shown that the 5' end of the plus strand of DNA is located in the DR2 region (Fig.1.2 f and g). If the primer oligoribonucleotide for plus strand synthesis comes from the 5' end of the RNA template, it has

to be upstream of the 5' end of the plus strand to act as a primer. Since the sequences in DR1 and DR2 are identical, it is presumed that the oligoribonucleotide primer must be transferred from DR1 to DR2 at the beginning of plus strand synthesis (Lien *et al.*, 1986, Will *et al.*, 1987). The synthesis of the plus strand of DNA thus begins near the DR2 region and the DNA elongates until it is close to the DR1 region at the 5'end of minus strand DNA (Fig.1.2 g). At this point, an intramolecular template switch occurs (Fig. 1.2 h). This template switch benefits from the short terminal redundant sequence of minus strand DNA that allows the short pieces of plus strand sequence to be stabilized at the 3' end of the minus strand (Fig. 1.2 h). After this, elongation of the plus strand can continue without interruption.

It has been found that completion of the plus strand synthesis is not necessary for maturation of the virus. Budding of virions takes place during plus strand DNA synthesis. It has been reported that RNA:DNA hybrids were detected in Dane particles from human plasma (Miller *et al.*, 1984). Thus the signal for secretion of the virus into the extracellular circulation may not be directly related to the initiation of second strand DNA synthesis. However, particles with RNA:DNA hybrids represent a small portion of the total virions. In most cases at least part of the plus strand synthesis is complete before virion budding.

1.5 Antiviral Therapy for HBV Infection.

1.5.1 Therapy for Chronic Hepatitis B Virus Infection.

Although there are millions of chronic HBsAg carriers in the world, currently there is no effective treatment for chronic hepatitis B viral infection. Attempts to treat chronic carriers have included administration of immunosuppressive and/or antiviral agents. For example, studies using corticosteroids, interferon and adenine arabinoside (araA) have given variable results. No therapy used alone, or in combination, has been able to produce a consistent benefit (Czaja, 1986, Hoofnagle and Jones, 1989). Immunosuppressive drugs, such as corticosteroids which are used for autoimmune chronic hepatitis ($HBsAg^-$), showed no therapeutic effect on chronic hepatitis B ($HBsAg^+$) with either short or long term treatment and in fact may be harmful (Lam *et al.*, 1981, Hoofnagle *et al.*, 1986b, Tygstrup *et al.*, 1986). Treatment with araA or its water soluble phosphorylated form, araA 5'-monophosphate (araAMP), was able to decrease the HBV DNA polymerase activity in the sera of some chronic hepatitis patients and, with one month treatment courses, seroconversion as well as inhibition of viral replication occurs in some patients (Weller *et al.*, 1982). The treatment with araAMP also clears the expression of HBcAg and improves the histological features of chronic active hepatitis in approximately one third of the patients treated

(Tyrrell *et al*, 1988). Unfortunately, other clinical trials have failed to demonstrate consistent beneficial effects of treatment with araAMP in chronic hepatitis B and treatment has been associated with severe neurotoxicity (Hoofnagle *et al.*, 1984, Weller *et al.*, 1985, Garcia *et al.*, 1987). Thus this agent was subsequently withdrawn from use in humans (Hoofnagle and Jones, 1989).

Numerous interferon trials have reported the use of interferon treatment for chronic hepatitis B (Davis and Hoofnagle, 1986, Perrillo, 1989a). These studies are difficult to compare because the individual trials differed in dose, length of treatment, and type and source of interferon. Nevertheless, the information obtained from these studies demonstrates that alpha interferon has consistently shown beneficial effects in chronic hepatitis B (Perrillo, 1989b, Lok *et al.*, 1989). It has been shown that approximately one third of patients respond to alpha interferon therapy with a sustained loss of viral replication markers from sera (Hoofnagle *et al.*, 1988, Perrillo *et al*, 1990). The effect of alpha interferon is dose-dependent, however, and treatment with higher doses is more frequently associated with serious side effects (Lok *et al.*, 1989). The best response is likely to be achieved when interferon acts in a dual fashion to augment the immune response to HBV as well as to inhibit viral replication. It generally requires a minimum of 3 months of treatment (Perrillo, 1989b).

In order to increase the efficacy of interferon, therapy with interferon in combination with other agents has been used in the treatment of chronic hepatitis B. Scullard *et al.* (1981) in a trial of interferon combined with araA reported that the combined treatment is better than that with either agent alone. A similar therapeutic trial reported by Garcia *et al.* (1987), however, showed that serious neurotoxicity resulted from araAMP and interferon used in combination. A controlled trial using "prednisone priming" (pretreatment with prednisone starting with large doses and tapering over 6 weeks) followed by recombinant alpha interferon suggested that this approach would increase the rate of response to alpha interferon therapy (Perrillo *et al.*, 1988). In this study, a sustained loss of HBV DNA was observed in 9 of 18 treated patients, HBeAg disappeared in eight and HBsAg became negative in four. In contrast, only 3 of 21 untreated control subjects underwent spontaneous HBeAg seroconversion and none lost HBsAg. The value of "prednisone priming" has not been substantiated in other studies (Perrillo *et al*, 1990) and most studies using interferon do not have a prednisone priming period. It has been demonstrated from clinical studies that characteristics of the patients, such as age of HBV acquisition, sex, lifestyle, level of ALT, level of HBV DNA, race and HIV seropositivity, are very important determinants for a positive response (Perrillo, 1989b).

1.5.2 Screening for Effective Antiviral Agents for Hepadnaviruses.

Hepadnaviruses have their own DNA polymerase and reverse transcriptase which offer targets for antiviral agents. Searching for compounds that are inhibitory in isolated preparations of viral polymerase has been a common method used to screen for anti-HBV drugs. Agents which have been tested include intercalating agents (Hirschman and Garfinkel, 1978), antiviral agents effective against retroviruses (Farranye *et al.*, 1989, Nordenfelt *et al.*, 1987) and nucleoside analogues (Tao *et al.*, 1988, Lofgren *et al.*, 1989). Some of these compounds have shown inhibitory activity on isolated core preparations containing DNA polymerase activity (Hirschman *et al.*, 1978), however when tested on patients these compounds have not produced corresponding antiviral activity (Bodenheimer *et al.*, 1983). These results suggest that inhibitors of viral polymerase detected by screening on isolated core particles containing DNA polymerase and reverse transcriptase activities will not necessarily be effective *in vivo*.

More effective methods of screening antiviral agents for activity against hepadnaviruses became available with the ability to grow these viruses in primary hepatocyte cultures or in hepatoma cell lines (Tuttleman, 1986b, Suzuki *et al.*, 1988, Price *et al.*, 1989, Ueda *et al.*, 1989). Among animal

hepadnaviruses, the DHBV system has been considered to be a good *in vitro* and *in vivo* model for primary screening (Zuckerman, 1987, Suzuki *et al.*, 1988, Kassianides *et al.*, 1989, Haritani *et al.*, 1989). Even though these screening procedures are relatively new, a few compounds have been identified which inhibit hepadnaviruses in hepatocyte cultures (Suzuki *et al.*, 1988, Lee *et al.*, 1989, Ueda *et al.*, 1989, Price *et al.*, 1989). The prospect of using these compounds as effective agents to treat chronic HBV infection in man is encouraging, but a considerable amount of work on mechanism of action, potential toxicity and efficacy *in vivo* remains to be done.

1.6 The Rationale of This Study.

As discussed previously, there is no satisfactory antiviral therapy for chronic hepatitis B infection at present. Although effective vaccines that can prevent the spread of HBV are available, the use of vaccines has not significantly decreased the carrier rate, even in highly industrialized countries which can afford this vaccine. Furthermore, vaccines will not benefit people who are already HBV carriers. More effective antiviral agents are required for the treatment of acute or chronic hepatitis B infection in humans.

Until recently the development of antiviral therapy was seriously hampered by the absence of cell culture methods to grow hepadnaviruses. In 1986, the technique of preparing primary hepatocyte cultures from DHBV infected ducks was established in the Fox Chase Cancer Institute in Philadelphia (Tuttleman *et al.*, 1986b). This provided a cell culture system which could be used to screen chemicals for antiviral activity against hepadnaviruses. Compounds which were effective *in vitro* could then be tested in DHBV infected ducks to determine if they were also effective *in vivo*.

At the time that *in vitro* screening began in our laboratory, no cell culture studies testing compounds for anti-HBV activity had been published. We began screening a number of compounds, including 2′,3′-dideoxynucleosides, for antiviral activity. Previous studies with these compounds had been limited to studies on the inhibition of human immunodeficiency and herpes viruses (De Clercq, 1987, Waqar *et al.*, 1984). We compared the antiviral activity of many compounds with the activity of araA, a compound known to have some anti-HBV activity. The results from our primary screening showed that two purine dideoxynucleosides, 2′,3′-dideoxyadenosine (ddA) and 2′,3′-dideoxyguanosine (ddG), were very potent inhibitors of DHBV replication whereas two pyrimidine 2′,3′-dideoxynucleosides, 2′,3′-dideoxycytidine (ddC) and 2′,3′-dideoxythymidine (ddT), had little or no inhibitory effect on viral replication (Suzuki *et al.*, 1988).

This presented us with an interesting challenge: to determine why the purine, but not the pyrimidine, 2′,3′-dideoxynucleosides analogues were potent inhibitors of hepadnaviruses.

The Objectives of this Study.

i. To determine the dose response curves for antiviral activity of 2′,3′-dideoxynucleosides in DHBV infected primary hepatocytes.

ii. To determine the *in vivo* efficacy of 2′,3′-dideoxynucleosides in Pekin ducks.

iii. To determine the mechanism of action of purine 2′,3′-dideoxynucleosides by:

a. examining their effects on DNA polymerase and reverse transcriptase activities.

b. examining their effect on DHBV RNA template synthesis and DHBV DNA synthesis in replicative core particles.

c. testing for binding of a purine dideoxynucleotide to the terminal protein.

CHAPTER 2.

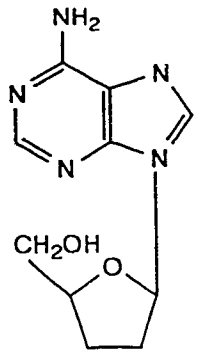
THE ABILITY OF 2',3'-DIDEOXYNUCLEOSIDES TO INHIBIT DUCK HEPATITIS B VIRUS: Antiviral Activity *in vitro* and *in vivo*.

INTRODUCTION

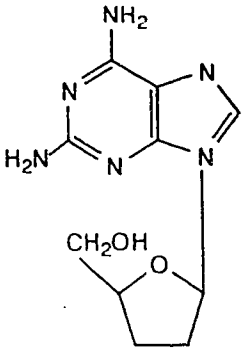
The initial screening studies done in our laboratory indicated that ddA and ddG were strong inhibitors of DHBV, whereas ddC and ddT had little or no effect on viral replication (Suzuki, *et al.*, 1988). This result was surprising since all of the dideoxynucleosides would have been expected to have approximately similar potency to inhibit DHBV DNA synthesis. The very potent antiviral activity of the purine 2',3'-dideoxynucleosides and the poor antiviral activity of the pyrimidine analogues required confirmation and further study by more carefully examining the antiviral activity of other purine and pyrimidine analogues. The antiviral activities of numerous 2',3'-dideoxynucleoside analogues were tested and compared in dose-response curves. The structures of the compounds which were studied are shown in figure 2.1. This work was completed in collaboration with Bonita Lee, a medical student who worked in our laboratory.

Figure 2.1.

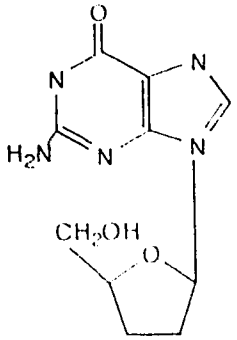
Molecular structures of some dideoxynucleosides.



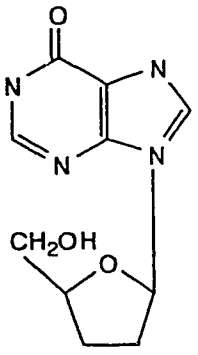
ddA



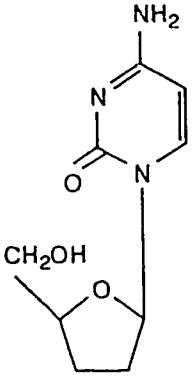
ddDAPR



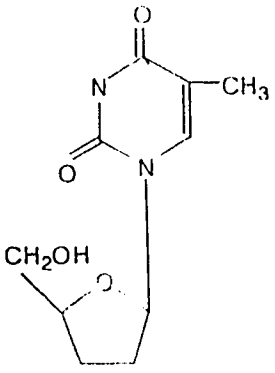
ddG



ddI



ddC



ddT

For many compounds, activity *in vitro* has not necessarily meant that these compounds have activity *in vivo*. For example, trisodium phosphononoformate showed an inhibitory effect on DHBV DNA polymerase *in vitro* (Nordenfelt *et al.* 1979), however, this compound gave no evidence of antiviral activity *in vivo* (Nordenfelt *et al.* 1982). Similar observations were made with suramin, which is reported to be an inhibitor of DHBV DNA polymerase *in vitro* (Tsiquaye and Zuckerman, 1985) and to be able to prevent virus spread by inhibiting infection by DHBV (Petcu, *et al.* 1988), but failed to suppress the HBV DNA polymerase in patients (Loke, *et al.* 1987). As another example, a number of intercalating agents had been demonstrated by Hirshman and Garfinkel (1978) to inhibit HBV DNA polymerase *in vitro*. Despite its potent inhibitory effect, *in vivo* studies failed to show antiviral activity against HBV in patients (Bodebgeimer, *et al.* 1983). For this reason, I am anxious to study the *in vivo* activity of purine 2',3'-dideoxynucleosides against DHBV.

The supplies of the 2',3'-dideoxynucleosides were obtained from the laboratory of Dr. M.J. Robins (Department of chemistry, Brigham Young University, Provo Utah84602) or Raylo Chemicals Ltd of Edmonton. Although ddG was the most promising compound, the chemical synthesis of ddG is much more difficult than the synthesis of 2,6-diaminopurine 2',3'-dideoxynucleoside (ddDAPR) or pyrimidine analogues. ddDAPR, which is readily deaminated to ddG in hepatocytes (personal

communication, Theresa Kitos), is easy to synthesize and the process could readily be "scaled up" to produce sufficient quantities for our *in vivo* studies. Hence, I elected to use ddDAPR, ddA and ddC in most of my *in vivo* studies.

MATERIALS and METHODS

Chemicals. 2′,3′-Dideoxyadenosine (ddA), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyguanosine (ddG), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxythymidine (ddT) were purchased from Pharmacia Canada Inc. for the *in vitro* screening. 2,6-diaminopurine 2′,3′-dideoxynucleoside (ddDAPR) for the *in vitro* work was synthesized by Morris J. Robins, Brigham Young University. The *in vivo* studies required larger quantities of ddA, ddC and ddDAPR. These larger quantities were purchased on contract from Raylo Chemicals, Edmonton, Canada.

Ducks. Fertilized Pekin duck eggs were purchased from Macey Foods Ltd., Brandon, Canada or Sunshine Chick Hatchery and Supplies Ltd., Edmonton, Canada. Newly hatched ducklings were infected by a 50 µl intravenous injection of serum containing DHBV. DHBV-infected ducklings were identified by dot hybridization of serum samples to detect DHBV-DNA 4-7 days after inoculation. Infected animals were raised separately from uninfected animals.

Hepatocyte Cultures and Drug Treatment. Primary culture of duck hepatocytes were prepared by a modification of the method described by Tuttleman, *et al.* (1986b). A duckling of 1- to 2-weeks of age was anesthetized with pentobarbital sodium (Nembutal). After the abdomen was opened, the portal vein was severed and the liver was

perfused via cardiac catheterization with 200 ml of minimum essential medium, Eagle (MEM Auto-Pow, Flow Laboratories, Inc.) containing 0.5 mM EGTA [ethylene glycol-bis(b-aminoethyl ether)-N,N,N´,N´-tetraacetic acid], 50 U/ml of penicillin G sodium and 10 µg/ml of streptomycin sulfate. The perfusion was continued with an additional 200 ml of the same medium containing 0.5 mg/ml of collagenase type IV (Sigma Chemical Co.)and 2.5 mM $CaCl_2$. The perfusion was performed in a horizontal laminar flow hood and media were kept at 37° C during the perfusion. When the liver became pale and soft (about 20 min), it was cut from the animal and removed to a 100 mm tissue culture dish containing L-15 media. The cells were dispersed in L-15 medium (Flow Laboratories, Inc.) containing 5% fetal bovine serum (FBS), 15 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 1 µg/ml of insulin (Sigma Chemical Co.), 10 µM hydrocortisone-hemisuccinate (Sigma Chemical Co.), 1.5 µg/ml of glucose, 50 U/ml of penicillin G sodium, 10 µg/ml of streptomycin and 25 U/ml of nystatin (culture medium, Tuttleman, *et al.* 1986b). The suspension of cells was filtered though a nylon mesh and centrifuged at 300 rpm (about 50 x g) for 10 minutes. The cell pellets were washed with culture medium 3 times by centrifugation at room temperature and the cells were suspended in approximately 10 times the pellet volume of culture medium and counted in a hemocytometer. Cells were seeded in 60-mm cell culture dishes in 4 ml of culture medium at approximately 2.5 x 10^6

cells/per dish. Cells were incubated at 37° C incubator. The day of the preparation of the hepatocyte cell cultures was counted as day 0. Media were changed every second day. Nucleoside analogues dissolved in 20 mM Tris-HCl (pH 7.5) were added to the hepatocyte culture media from day two onwards.

***In vivo* drug treatment.** Persistent DHBV infection was confirmed in 4- to 6-week-old ducklings by dot hybridization prior to treatment. Nucleoside analogues were dissolved in 20 mM Tris-HCl (pH 7.5) and 0.72% NaCl, filtered though 0.45 μm filters and stored at -20° C in aliquots. Ducks were weighed twice a week and treated with placebo (20 mM Tris-HCl, pH 7.5 and 0.72% NaCl, 2 ml/kg), ddA (10 mg/kg), ddDAPR (10 mg/kg) or ddC (10 mg/kg) given twice daily (bid) by intramuscular (i.m.) injection. Blood samples were collected every 3-7 days from veins of duck legs during the treatment period and in some cases weekly for 1-4 weeks post-treatment. The sera were stored at -20° C.

Plasmid DNA and preparation of the radioactive probe. Plasmid pDH010-DHBV contains a full sized DHBV genome at the EcoRI site. *Escherichia coli* harboring plasmid pDH010-DHBV was a generous gift from Dr. J. W. Summers (Dept. Cell Biol., U. of New Mexico School of Medicine, Albuquerque, NM 87131). The plasmids were prepared using the CsCl gradient method as described by Maniatis, *et al.* (1982). Purified plasmids containing the DHBV DNA sequence were

labelled with [α-^{32}P]dCTP by nick translation (Nick Translation Kit, Amersham).

Dot hybridization. Duck serum samples (10 µl) were loaded onto a nylon membrane (Hybond-N, Amersham) using a Bio-Dot ™ microfiltration apparatus (BIO-RAD). The membrane was placed on a Whatman 3 MM filter saturated with denaturation buffer (0.5 M NaOH and 1.5 M NaCl) and left at room temperature for 30 minutes before being placed on a Whatman 3 MM filter saturated with neutralization buffer (1 M Tris-HCl and 1.5 M NaCl pH 8.0) for another 30 minutes. The DNA was fixed on the membrane by exposure to UV light (short-wavelength, 254 nm) for 2-5 minutes. Hybridization was performed as described by Maniatis, *et al.* (1982). The membrane was prehybridized for 4-16 hours at 42° C in 50% formamide, 5x SSPE (20x SSPE contains 3.6 M NaCl, 200 mM NaH_2PO_4, pH 7.5 and 20 mM EDTA), 5x Denhardt's solution (50 x Denhardt's solution contains 10 g of Ficoll, 10 g of polyvinylpyrrolidone, 10 g of bovine serum albumin per liter), 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. Hybridization was continued under the same conditions in the presence of [α-^{32}P] labelled DHBV DNA probe (1 x 10^6 cpm/ml) for 16 hours. After hybridization, the membrane (70 cm^2) was washed in 200 ml of 1x SSC (20x SSC contains 3 M NaCl, 0.3 mM Na Citrate pH 7.0), 0.1% SDS at 65° C for 4 times, 30 min each. The membrane was washed again in 200 ml of 1x SSC at room temperature for 30 minutes. The membrane was

autoradiographed at -70° C using XAR Kodak film and an enhancer screen for overnight.

DNA from hepatocyte cell cultures. The preparation of DNA was performed as previously described by Tuttleman, *et al.* (1986b). In brief, the medium was removed from cell culture dishes and 1 ml of lysis solution containing 0.2% SDS, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 5 mM EGTA and 150 mM NaCl was added to each dish. After gentle shaking at room temperature for about 20 minutes, the cell lysate was transferred to a test tube and 0.5 mg/ml of pronase E (type E, Sigma Chemical Co.) was added. The lysate was digested at 37° C for 1.5 to 2 hours and deproteinized by extraction with an equal volume of phenol (saturated with 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA and 0.1% 8-hydroxyquinoline). Ammonium acetate was added to the aqueous phase to yield a final concentration of 0.2 M and the nucleic acids were precipitated with 2 volumes of cold ethanol. The pellet of nucleic acid was washed with 70% ethanol and dried. The DNA was dissolved in 100 µl of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (TE buffer) and one quarter of each was used in the dot hybridization studies.

Dose response Curve. DNA was extracted from hepatocyte cultures and the relative amounts of DHBV DNA present could be approximated by dot hybridization. However to obtain quantitative data for the dose-response curves, the "dots" were cut out from the nylon filters and the

radioactivity of each was counted in a scintillation counter. The antiviral effect was expressed as a percentage of radioactivity in the treated cultures relative to that in the untreated control. The amount of radioactivity in untreated-infected hepatocytes was considered to be 100%.

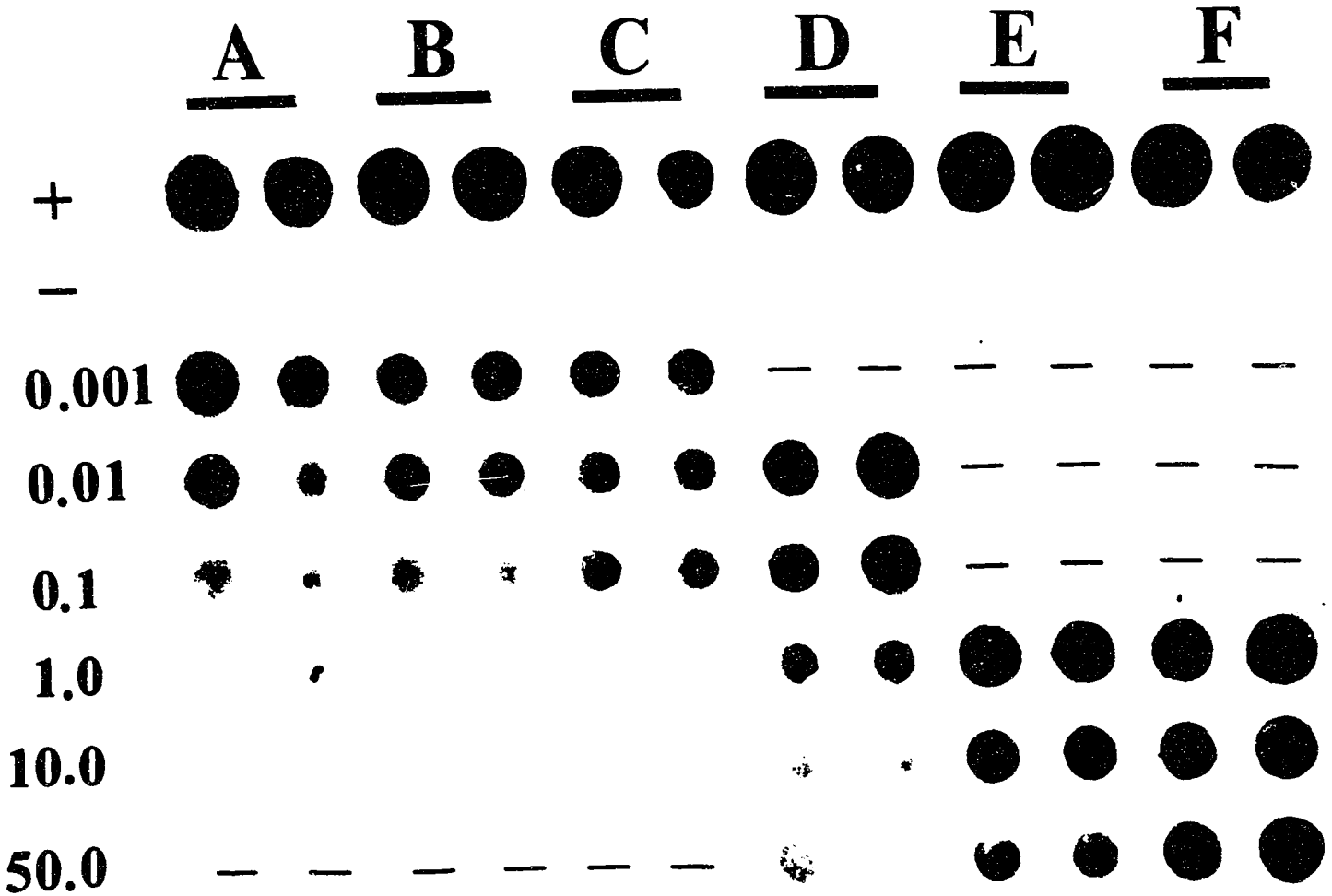
RESULTS

2.1 *In Vitro* Antiviral Activity of 2',3'-Dideoxynucleoside Analogues Against DHBV.

For these experiments, I selected a range of concentrations from 0.01 µg/ml to 50 µg/ml for each of the 2',3'-dideoxynucleosides to be tested. Hepatocyte cultures prepared from DHBV-infected ducklings were treated with nucleoside analogues as described above. At the end of 18 days, the total DNA (cellular and viral) was harvested from each plate. Figure 2.2 shows the results of the dot hybridizations with the extracted DNA samples. Each sample was done in duplicate. These results showed that ddDAPR, ddG and ddA (Fig. 2.2 samples A to C) were effective inhibitors of DHBV DNA synthesis and allowed clearance of the virus from the cells. At very low concentrations (0.01-0.1 µg/ml), there was partial clearance of DHBV-DNA from the cells treated with ddDAPR, ddG and ddA. At higher concentrations (≥1.0 µg/ml), there was nearly complete clearance of viral DNA from the hepatocyte cultures treated with ddDAPR, ddG and ddA. The other purine 2',3'-dideoxynucleoside, namely ddI, permitted only partial clearance of DHEV-DNA from the cells compared with the above compounds since it showed this effect only when used at concentrations higher than 1.0 µg/ml (Fig. 2.2 sample D). However, ddI was more effective than either of the pyrimidine 2',3'-dideoxynucleosides tested, namely ddC

Figure 2.2.

Dot hybridization of DNA extracts from DHBV-infected hepatocyte cultures treated in duplicate with various concentrations of six different 2',3'-dideoxynucleosides. DHBV-infected hepatocyte cultures were treated with drugs from day 2 to day 18 and total DNA of each plate was extracted after treatment. Each dot represented a quarter (25 µl) of DNA extracted from one 60 mm plate and each sample was done in duplicate. Positive controls (+) and negative controls (-) were DNA extracted from DHBV-infected hepatocyte cultures with no treatment and uninfected hepatocyte cultures, respectively. Drug concentrations are shown on the left side in µg/ml and the columns show DNA samples treated with ddDAPR (A), ddG (B), ddA (C), ddI (D), ddC (E), and ddT (F), respectively. Dashes represent concentrations that were not done.


A
B
C
D
E
F
+
−
0.001
0.01
0.1
1.0
10.0
50.0

and ddT (Fig. 2.2 samples E and F). Neither of ddC nor ddT permitted significant clearance of viral DNA at concentrations of 1.0 to 10 µg/ml, although ddC does show some effect on DHBV-DNA clearance at 50 µg/ml.

The concentration that resulted in a 50% decrease in the viral DNA content of infected hepatocyte cultures (IC_{50}) was determined for each of these compounds from the dose response curves (Fig. 2.3). ddDAPR and ddG were the most potent agents permitting DHBV-DNA clearance among the compounds that were tested. Both of these compounds have IC_{50}s of 0.07 µg/ml. ddA also permitted clearance of viral DNA from the cells at a low concentration, with an IC_{50} of 0.12 µg/ml. ddI was a relatively weak agent with an IC_{50} of 1.5 µg/ml, and even at high concentrations, failed to permit complete clearance of DHBV-DNA from hepatocytes. ddC and ddT did not permit clearance of viral DNA from hepatocytes.

2.2 *In Vivo* Testing of 2',3'-Dideoxynucleoside Analogues

Twelve 6-week old ducks infected with DHBV were divided into three groups of four animals each for an *in vivo* study to compare the effectiveness of ddA and ddDAPR. The animals were treated with placebo (group A), ddDAPR at 10 mg/kg (group B) or ddA at 10 mg/kg (group C) given twice daily for three weeks in i.m. injections. Sera were collected weekly and analyzed by dot hybridization as shown in figure 2.4.

Figure 2.3.

Dose-response curves for 2',3'-dideoxynucleosides in hepatocyte cultures. DHBV-infected hepatocyte cultures were treated with drugs as described in figure 2.2. The "dot" was then cut out and counted in a liquid scintillation counter. Each point represents the mean of duplicate samples. Viral replication is expressed as the amount of radioactivity from treated cells relative to that from untreated DHBV-infected cells (100%). Symbols: ddDAPR, ; ddG, ; ddA, ; ddI, ; ddC, ; ddT, .

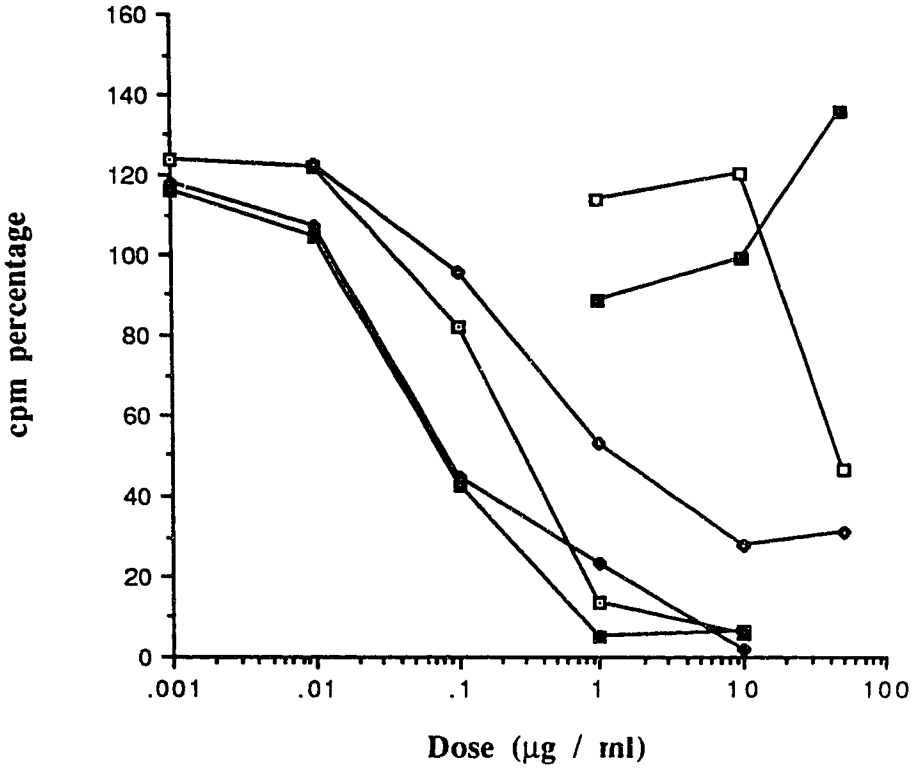
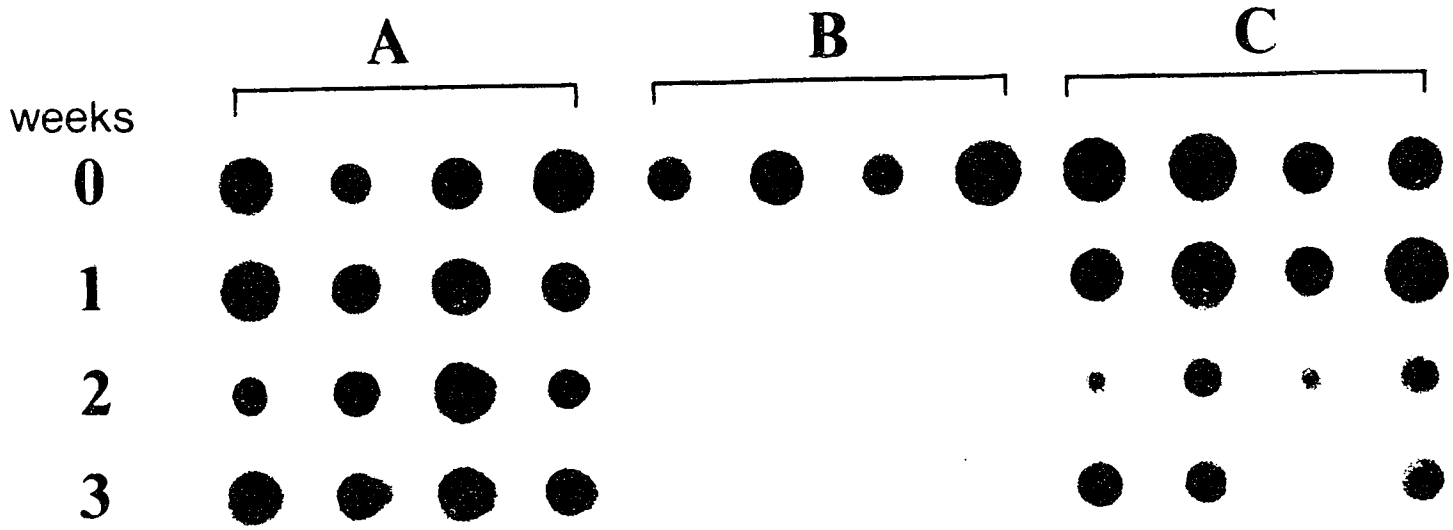

160
140
120
100
80
60
40
20
0
cpm percentage
.001
.01
.1
1
10
100
Dose (μg / ml)

Figure 2.4.

Dot hybridization of serum samples from ducks treated with placebo, ddDAPR , or ddA. DHBV-infected 5-week-old ducks were treated with placebo (20 mM Tris-HCl, pH 7.5 and 0.72% NaCl, 2 ml/kg, group A), ddDAPR (10 mg/kg, group B), or ddA (10 mg/kg, group C) i.m. bid. Four ducks received each treatment and sera were collected pretreatment and during treatment. Each dot represents the DHBV-DNA in 10 µl of serum. Serum samples in row 0 were taken pretreatment. Row 1, 2, and 3 contain serum samples after one, two and three weeks of treatment.

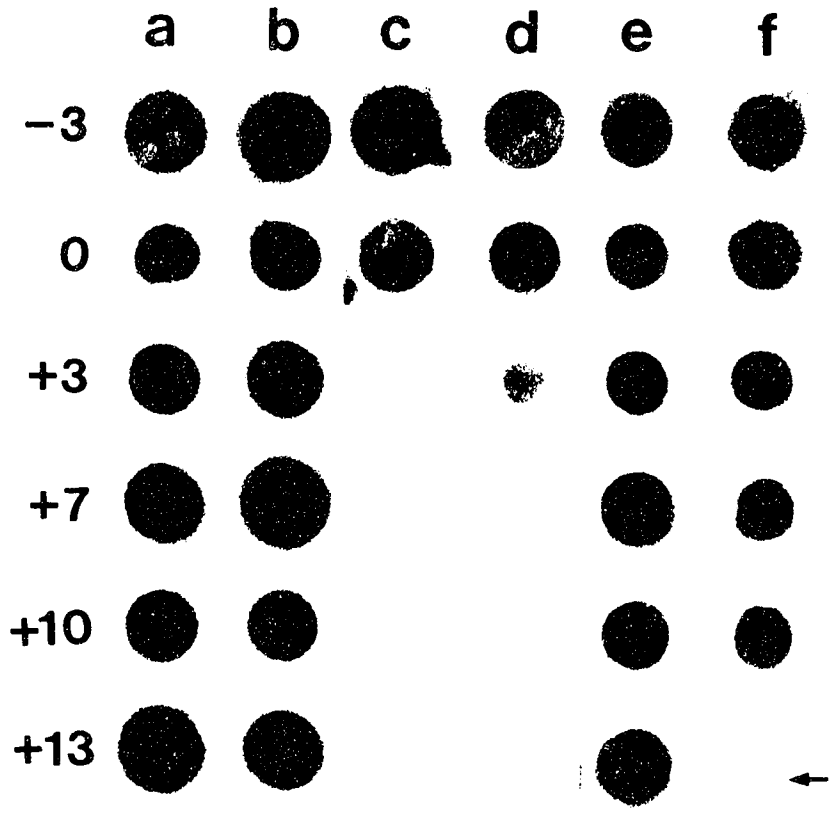

A
B
C
weeks
0
1
2
3

DHBV infection of the ducks was evident from the high levels of DHBV DNA in the sera of all ducks prior to treatment. These levels of DHBV DNA in the placebo-treated animals remained unchanged (Fig. 2.4, group A). Treatment with ddDAPR, the most effective anti-DHBV drug *in vitro*, resulted in almost complete clearance of DHBV DNA from the sera of all four animals within one week (Fig. 2.4, group B). Treatment with ddA was much less effective than ddDAPR. There was a decrease in the amount of DHBV DNA after two weeks of treatment with ddA, but a significantly higher amount of DHBV DNA remained in the sera after three weeks of treatment (Fig. 2.4, group C). Although the inhibitory effect of ddA *in vitro* was very close to that of ddDAPR (Fig.2.2.), it was not nearly as good an inhibitor as ddDAPR *in vivo*.

I also compared the *in vivo* inhibitory effect of the pyrimidine 2',3'-dideoxynucleoside, ddC with ddDAPR. Six ducks infected with DHBV were used in this study and divided into three groups of two animals each. Ducks were treated with placebo, ddDAPR at 10 mg/kg or ddC at 10 mg/kg, twice daily by intramuscular injection. Sera from these ducks were taken every 3 days and examined by dot hybridization. The results of the dot hybridization are shown in figure 2.5. Again, placebo treatment had no inhibitory effect on the production of DHBV (Fig. 2.5, samples a and b). Rapid clearance of DHBV from sera of ddDAPR-treated ducks was observed (Fig. 2.5, samples c and d). Treatment with ddC

Figure 1.

Dot hybridization of serum samples from ducks treated with placebo, ddDAPR, or ddC. DHBV-infected ducks of 4-week-old were treated with placebo (20 mM Tris-HCl, pH 7.5 and 0.75% NaCl, 2 ml/kg, columns a and b), ddDAPR (10 mg/kg, columns c and d), or ddC (10 mg/kg, columns e and f) i.m. bid. Each duck received 2 weeks of treatment and sera were collected before and during treatment. Each dot represents the DHBV DNA in 10 µl of serum. Numbers on the left indicate days of sample collection. Sera collected before treatment are labelled as -3 and 0 days, whereas sera collected during the treatment period are shown as +3, +7, and +10 days. Sera in each column were taken from the same animal. The arrow indicates that no sample was available because the duck had died on day 12.

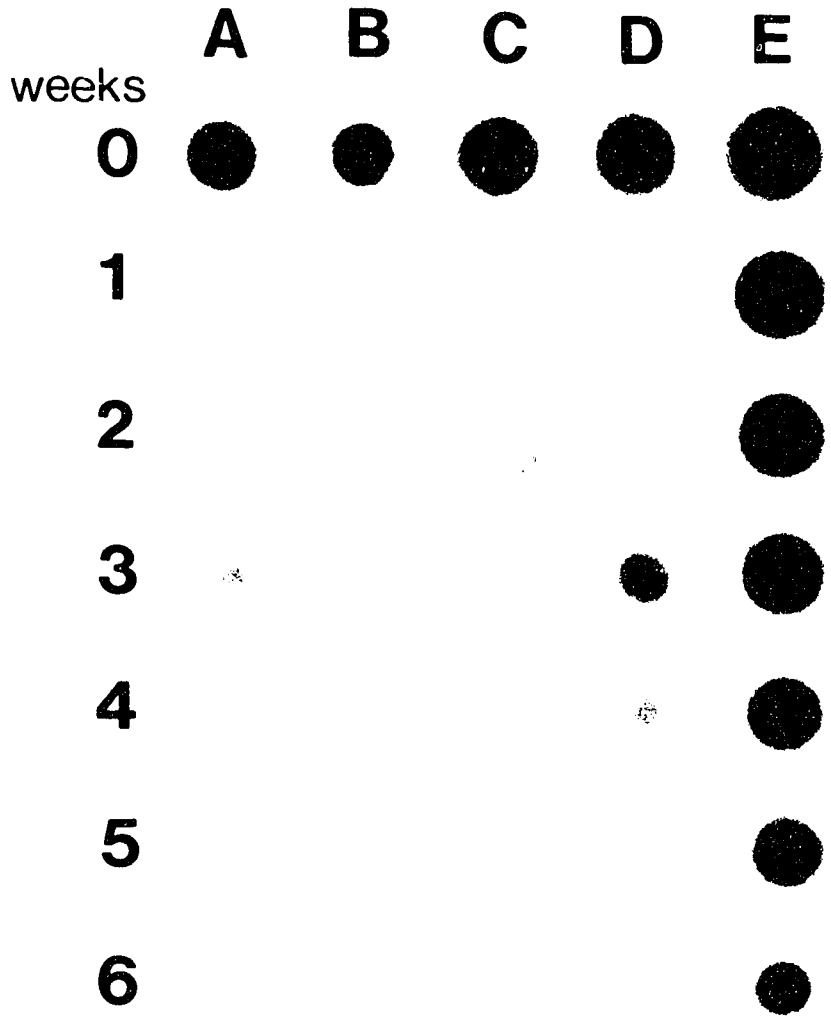

a
b
c
d
e
f
−3
0
+3
+7
+10
+13

resulted in very little inhibition of DHBV replication (Fig. 2.5, samples e and f). This result indicated that ddC, which was not an effective inhibitor of DHBV in hepatocyte culture, did not inhibit DHBV *in vivo* either. Furthermore, in my studies which have been repeated twice more, ddC was considerably more toxic than ddDAPR. I have noted that ducks treated with ddC at 10 mg/kg twice daily frequently died between 2-3 weeks into treatment. Ducks receiving ddDAPR did not show any noticeable toxicity in comparison to ducks treated with placebo.

The rapid clearance of DHBV from sera of ddDAPR-treated ducks led me to question whether this clearance of the virus would be sustained after cessation of the treatment. I have carried out another experiment in a group of five ducks. Four of them were treated with ddDAPR at 10 mg/kg twice daily i.m.and one received placebo. The treatment lasted for two weeks and sera were collected weekly from a pretreatment sample to one at the end of six weeks after the first injection. As shown in figure 2.6 samples A to D, viral DNA could no longer be detected in the sera of four ddDAPR-treated ducks within one week and the sera remained clear during the treatment period. After the treatment was stopped, there was a rebound of viral DNA in the sera during the following week. The level of viral DNA in the sera of ddDAPR treated ducks was much lower than it had been before the treatment, however. This suggests that the subsequent

Figure 2.6.

Dot hybridization of serum samples from four ducks treated with ddDAPR and one with placebo (E). DHBV-infected ducks of 6-week-old were treated with ddDAPR (10 mg/kg, columns A to D) or placebo (20 mM Tris-HCl, pH 7.5 and 0.72% NaCl, 2 ml/kg, column E) i.m. bid. Sera were collected before the treatment (week 0), during the treatment (week 1 and 2) and post treatment (week 3 through 6). Each dot represents the DHBV DNA in 10 µl of serum. Numbers on the left indicate the weeks of serum collection.


A
B
C
D
E
weeks
0
1
2
3
4
5
6

production of virions was affected by the two weeks of ddDAPR treatment for at least 4 weeks.

DISCUSSION

In vitro screening for anti-DHBV activity of 2',3'-dideoxynucleosides in this study once again demonstrated that the purine 2',3'-dideoxynucleosides led to effective clearance of DHBV-DNA from hepatocytes, whereas none of the pyrimidine analogues were effective. Among the purines, the 2',3'-dideoxyguanosine analogues, namely ddDAPR and ddG, appeared to be the most effective agents. Both ddDAPR and ddG exhibited the lowest IC_{50} values, indicating that they are the most potent agents permitting DHBV-DNA clearance. Studies of the metabolism of ddDAPR, which have been conducted in our laboratory (unpublished data, Theresa Kitos), have demonstrated that ddDAPR is rapidly deaminated to ddG in hepatocyte cultures as well as in the duck. The other purine 2',3'-dideoxynucleosides (ddA and ddI) also promoted DHBV-DNA clearance from the hepatocyte cultures although ddI was a relatively weak agent.

The activity of purine 2',3'-dideoxynucleosides, especially dideoxyguanosine analogues, against DHBV replication suggested that the clearance of viral DNA from hepatocytes occurs by a mechanism which favors the dideoxyguanosine analogues. These results were surprising since most 2',3'-dideoxynucleosides are considered to be inhibitors of reverse transcriptase and previous studies on the inhibition of the reverse transcriptase of retroviruses

have not shown this marked preference for specific 2',3'-dideoxyguanosine analogues (Waqar, *et al.*,1984, De Clercq, 1987,). This suggests that the mechanism of inhibition of DHBV replication by ddDAPR and ddG might not be analogous to the inhibition of retroviruses by other 2',3'-dideoxynucleoside analogues. The mechanism of action of the purine 2',3'-dideoxynucleosides was examined in more detail as will be described in Chapter 4.

The *in vivo* testing is a very important part of antiviral screening. It not only examines the inhibitory effect of the compound *in vivo*, but also helps to establish the applicability of *in vitro* screening to *in vivo* activity. Studies in our laboratory have shown that acute toxicity of ddDAPR was at least >200 mg/kg when it was tested in 100 mg/kg and 200 mg/kg doses in mice (D. Gong, unpublished data). I selected two doses, 5 mg/kg and 10 mg/kg, given intramuscularly (i.m.) every 12 hours for my preliminary experiments in ducks. I noted that 5 mg/kg of ddDAPR given i.m. twice daily failed to clear the virus from the sera of treated animals in one week (data not shown), whereas a dose of 10 mg/kg of ddDAPR given i.m. twice daily cleared the virus in one week. Based on the results of these preliminary studies, I decided to compare the *in vivo* activity of ddDAPR, ddA and ddC at 10 mg/kg given i.m. twice daily.

The results of *in vivo* studies comparing the antiviral activity of ddDAPR, ddA and ddC have shown that the

dideoxyguanosine analogue, ddDAPR, was the most effective inhibitor of DHBV *in vivo*. This correlated well with the *in vitro* screening results. However, ddA which was relatively effective *in vitro*, showed a much lower inhibitory effect on DHBV replication *in vivo*. There are a few possible explanations for the decreased antiviral effect of ddA *in vivo*. The most likely possibility is that ddA is more easily deaminated to ddI *in vivo* than it is *in vitro*. As shown in the dose-response curves *in vitro*, ddI was the least effective inhibitor among all the purine dideoxynucleosides tested. If deamination of ddA occurs rapidly *in vivo*, the activity of ddA *in vivo* would not be the same as ddA *in vitro*. Rather, it is more likely to behave like ddI. Other factors may also contribute to this decreased antiviral activity of ddA *in vivo*. For example, ddA may not be phosphorylated as readily as ddG *in vivo*, or ddA may be more rapidly cleaved to the purine base and dideoxyribose than ddDAPR.

The results of *in vivo* testing of ddC in comparison with ddDAPR match very well with the predictions from the results of the *in vitro* screening. Not only did ddC not show any antiviral activity against DHBV replication *in vivo*, but treatment was associated with considerable toxicity. Although the reason for the sudden death in the animals treated with ddC remains unknown, it is clear that animals

could not tolerate prolonged treatment with ddC at 10 mg/kg,i.m. twice daily.

The reappearance of the viral DNA in sera of ducks after stopping treatment was not surprising since inhibition of viral DNA replication does not necessarily indicate the complete clearance of virus from hepatocytes. The nuclei of cells infected with hepadnaviruses contains cccDNA which could be relatively stable. In order to cure an animal of the virus, I need considerably more information about both the intracellular pools of the intermediates in the virus replicative cycle,and the optimal conditions for treatment with an agent such as ddDAPR. This part of my study is presented in Chapter 3.

Previous studies have found that results from the inhibition of viral DNA polymerase could not be extrapolated to a demonstration of antiviral activity of these compounds *in vivo* (Nordenfelt, *et al.*, 1979, Nordenfelt *et al.*, 1982, Hirshman and Garfinkel, 1978, Bodenheimer *et al.*, 1983, Tsiquaye and Zuckerman, 1985, Loke *et al.*, 1987). It is important to note that my testing of antiviral activity has been done within the same species. The activity in the duck hepatocyte culture has some predictive value *in vivo* for the duck. If a unique mechanism of action for the purine 2',3'-dideoxynucleoside analogues against hepadnaviruses can be proven, then it is possible that screening in DHBV-infected duck hepatocytes may also be predictive of antiviral activity

for other hepadnaviruses *in vivo*. When I began my studies, there was no cell culture system that I could use to study the antiviral activity of compounds against the human hepadnavirus. However recently, several investigators have produced HBV-transfected hepatoma cell lines which can be used for this purpose. The results of studies done so far have been quite variable, for example, ddA is active against HBV HepG-2 cells (Dorothy Tovell, Dr. Tyrrell's lab. and personal communication, Brent Korba, NIH) but inactive against HBV in hepatoma cell line HB611 (Ueda *et al.* 1989). It is unlikely that this represents differences in HBV strains, but more likely differences in the metabolism of ddA in different cell lines. Therefore it remains to be determined how useful the antiviral studies done in transfected tumor cells will be in predicting the effect of a given compound on HBV infection *in vivo*. Will the infected hepatocyte from the duckling be a better or worse screening system than the transfected hepatoma cell lines? This question remains to be answered.

CHAPTER 3.

STUDIES ON THE HALF LIVES OF DHBV DNA.

INTRODUCTION

Although I have shown that ddDAPR is an effective inhibitor of DHBV *in vivo* (Chapter 2), these experiments were performed without determining the best conditions for the use of this compound. In order to achieve the maximal inhibitory effect of ddDAPR on viral replication, which may be needed to cure a persistent viral infection, I need to know some important biological properties of the virus as well as the optimal condition for the use of ddDAPR in the *in vivo* treatment of carrier animals. To date, there is no available information about the half lives of any of the forms of the DNA of the hepadnaviruses in their biological hosts. The experiments described in this Chapter were designed to determine the half life of viral DNA in the serum as well as the half life of cccDNA in the hepatocyte nucleus. These parameters will provide me with the basic information required to design the protocol for an attempt to eliminate the virus in chronic infections. The effect of age of the animals on the rate of clearance of DHBV DNA from their sera with ddDAPR treatment will also be presented.

The determination of the half life of DHBV DNA (virion) in the serum is relatively simple because one animal can be used for repeated sampling. However, the determination of the half life of cccDNA in the hepatocytes nucleus is more complex. I thought of trying to use the same animal and determining the amount of cccDNA in sequential liver biopsy samples. However this procedure has several potential pitfalls. Firstly, the percentage of the hepatocytes infected during a persistent infection can vary from one animal to another (Dr. L. Jewell, personal observations). Secondly, the infection in the liver can be quite focal and biopsies may not be very reliable when quantity is most important. Thirdly, I found on several attempts that the amount of tissue obtained by a liver biopsy was not sufficient to allow me to do a quantitative extract of DHBV cccDNA. For these reasons, I decided to treat a larger number of animals and use samples from three to four animals for each time point.

I originally treated animals with ddDAPR at 10 mg/kg given i.m., twice daily and noted clearance of the virus from sera in approximately one week as described in Chapter 2. I also tested a dose of 20 mg/kg i.m. bid and noted clearance at approximately the same rate. I concluded therefore that 20 mg/kg i.m. bid would be above the maximal effective dose and could be used to determine the half lives of the viral DNA in the serum and the cccDNA in the liver. However, Dr.

Mang Ma in our laboratory has shown the inhibitory effect persists for approximately 8 hours post treatment. Therefore I repeated these experiments with a treatment regime of 20 mg/kg of ddDAPR given i.m., every 8 hours (q8h).

I also decided to use congenitally infected ducklings since I had observed that these animals were less likely to show spontaneous clearing or wide variations from one animal to another in the amount of virus present. There was one further problem in these studies. It appears that DHBV DNA clearance in newly hatched ducklings is much less sensitive to ddDAPR treatment than in animals that are >3 weeks of age. To avoid the problem of variable response in young ducklings, all the animals used in my later study were 5 weeks of age.

MATERIALS AND METHODS

Animals: Pekin ducks were raised in two colonies at the University of Alberta farm at Ellerslie, Alberta. One colony consisted of uninfected ducks and the other consisted of ducks that were congenitally infected with DHBV. Eggs obtained from these two colonies were hatched in the laboratory in a Cyclonic incubator (Marsh Manufacturing, Inc., Garden Grove, California 92641). The eggs were incubated for 28 days at 37° C in a humidity controlled environment.

Treatment: Initially I used 24 congenitally infected ducklings and treated these animals, beginning 12 hours after hatching, with ddDAPR at 20 mg/kg i.m., bid. Four animals were sacrificed at weekly intervals and livers of the animals were quick frozen in liquid nitrogen and stored at -70° C. The cccDNA was extracted from liver samples as described below.

This experiment was repeated using 5-week-old ducklings. Thirty of these animals were congenitally infected and one from the uninfected colony served as a negative control for the cccDNA preparation. In this experiment the serum half life of DHBV DNA, presumably the half life of genomic DNA in virion, was also measured. These ducklings were treated with ddDAPR at 20 mg/kg i.m., every 8 hours (q8h) for 15 days. Sera were removed from 6 animals pretreatment and at every 8

hours for 72 hours to determine the serum half life of DHBV DNA. Animals treated with ddDAPR were sacrificed at 1, 2, 3, 6, 9, 12 and 15 days. Animals treated with placebo (20 mM Tris HCl, pH7.5 and 0.72% NaCl, 2 ml/kg) i.m.,q8h were sacrificed at 0, 6 and 15 days. The livers were stored as described above.

Extraction of Cellular and cccDNA From Liver Tissue. Duck liver samples of 0.5 g were cut into small pieces and homogenized in a Dounce homogenizer in 2.5 ml of lysis buffer containing 0.4 M EDTA and 2% N-lauroylsarcosine sodium, pH 9.5. This high pH lysis solution will inhibit any nuclease activity and prevent the generation of nicks in the cccDNA. The homogenized solution was diluted with 5 ml of 0.10 M Tris HCl pH 8.0 to reduce its density, and immediately extracted twice with phenol and once with chloroform. The DNA was precipitated from the aqueous phase by the addition of NaOAc to 0.3 M and 2 volumes of ethanol. The DNA in the pellet was dissolved in TE buffer and treated with RNase A 0.1 µg/ml for 30 minutes at 37° C. After phenol extraction, the DNA was precipitated again and dissolved in 1 ml of 10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA. The chromosome DNA in the solution was sheared by passing through 26G,1/2 inch size needle 8 times. Samples were stored at -20° C.

DHBV DNA Probe and DNA Hybridization. Preparation of DHBV DNA probes and dot hybridizations were performed as described in Chapter 2. For Southern transfer and

hybridization, DNA samples were electrophoresed in a 1% agarose gel with either TBE buffer (20x TBE is 1 M Tris base, 1 M boric acid and 20 mM EDTA) or TAE buffer (40 mM Tris acetate, pH 7.5 and 2 mM EDTA). After electrophoresis, the gel was treated with acid (0.25 N HCl) for 10 minutes and with alkali (0.5 M NaOH, 1.5 M NaCl) for 30 minutes at room temperature with gentle agitation. The gel was neutralized in a solution containing 1.5 M NaCl and 1 M Tris HCl, pH 8.0, at room temperature for 30 minutes with gentle agitation. The DNA fragments separated on the agarose gel were transferred onto a nylon membrane with 20x SSC overnight as described by Maniatis, *et al.* (1982). DNA was fixed onto the nylon membrane by exposure to UV light for 2-5 minutes. Hybridization of an [α-^{32}P] labelled DHBV DNA probe to viral DNA on the membrane was performed as described for the dot hybridization in Chapter 2.

Determination of the Half Life of DHBV DNA in Sera. Sera were taken from the treated ducks initially and at every 8 hours for 72 hours. An aliquot of 10 µl of serum from each duck was applied to the nylon filter for hybridization. After hybridization, the dots were cut from the filter and the radioactivity present in each was determined by liquid scintillation counting. The amount of DHBV DNA in sera was expressed as a percentage of radioactivity present in dot blots of sera from DHBV-infected ducks relative to that present in ducks before initiating

treatment (100%). The time to 50%, 25%, 12.5% and 6.25% of the pretreatment level was used to calculate each of 4 half lives. The DHBV DNA half life was determined to be the mean of these four values.

Determination of the Half Life of DHBV cccDNA in Liver Tissue. The half life of DHBV cccDNA has been examined using dot blot hybridization or Southern blot hybridization followed by counting radioactivity on nylon filters. DNA samples assayed by dot hybridization were applied in 10 µl/5 mg of liver tissues/dot and the radioactivity of each dot was counted as described previously. DNA samples extracted from 5-week-old ducklings were also examined by Southern blot hybridization in 20 µl/10mg of liver tissue/well. After hybridization, the cccDNA band was cut and the radioactivity of each was quantified.

RESULTS

3.1 The Half Life of DHBV DNA (Virion DNA) in Serum.

The half life of DHBV DNA in serum was assayed in 5-week-old ducks. The DHBV DNA level in sera of ducks treated with ddDAPR decreased rapidly as shown in figure 3.1. There was a dramatic decrease in the level of DHBV DNA by 2 days of treatment. The half life of DHBV DNA in sera in the presence of ddDAPR, calculated from this graph by measuring four half lives (50%, 25%, 12.5% and 6.25%) and calculating a mean half life from these four, was found to be 14 hours. The similar experiments have been done by the treatment of ddDAPR at 20 mg/kg i.m. bid in the same age of ducks, and the values for the half life of DHBV DNA in sera ranged from 11 to 14 hours.

3.2 The Half Life of DHBV cccDNA in Liver Tissue.

DNA (10 µl/5 mg of liver tissue) extracted from livers of newly hatched ducklings was applied to nylon filters and assayed by dot hybridization. The results from newly hatched, congenitally-infected ducklings are shown in figure 3.2. The decrease of cccDNA from liver tissue in this group of animals was surprisingly slow. From the curve in figure 3.2, the half life of cccDNA was measured to be 1.3 weeks. I collected sera from these animals on a weekly basis and noted that treatment of ducks with ddDAPR at 20 mg/kg i.m. bid failed to clear DHBV from the sera of these animals even

Figure 3.1

DHBV DNA, measured at 8-hour intervals, in sera of ducks treated for 72 hours with ddDAPR at 20 mg/kg i.m. q8h. Each point represents the mean value from six animals and bars represent the standard deviation. The relative amounts of DHBV DNA in sera are expressed as the percentage of radioactivity present in dot blots of sera from DHBV-infected ducks with treatment, relative to that present in ducks before initiating treatment. Each 10 µl serum sample was dotted onto a nylon filter for dot hybridization and then cut out for quantitation by scintillation counting.

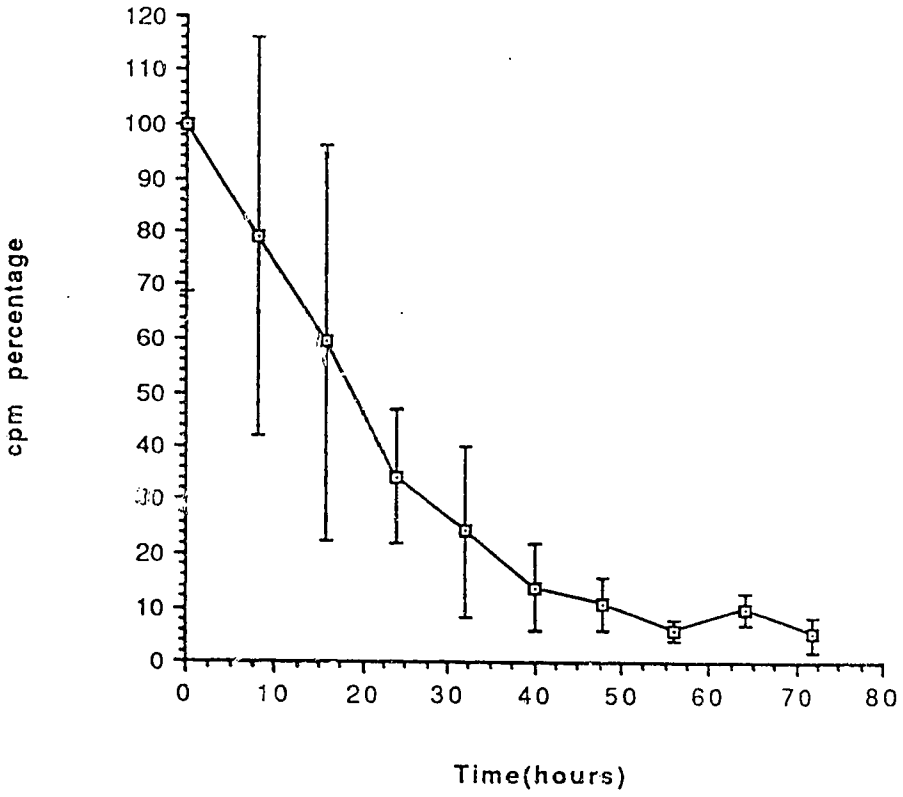
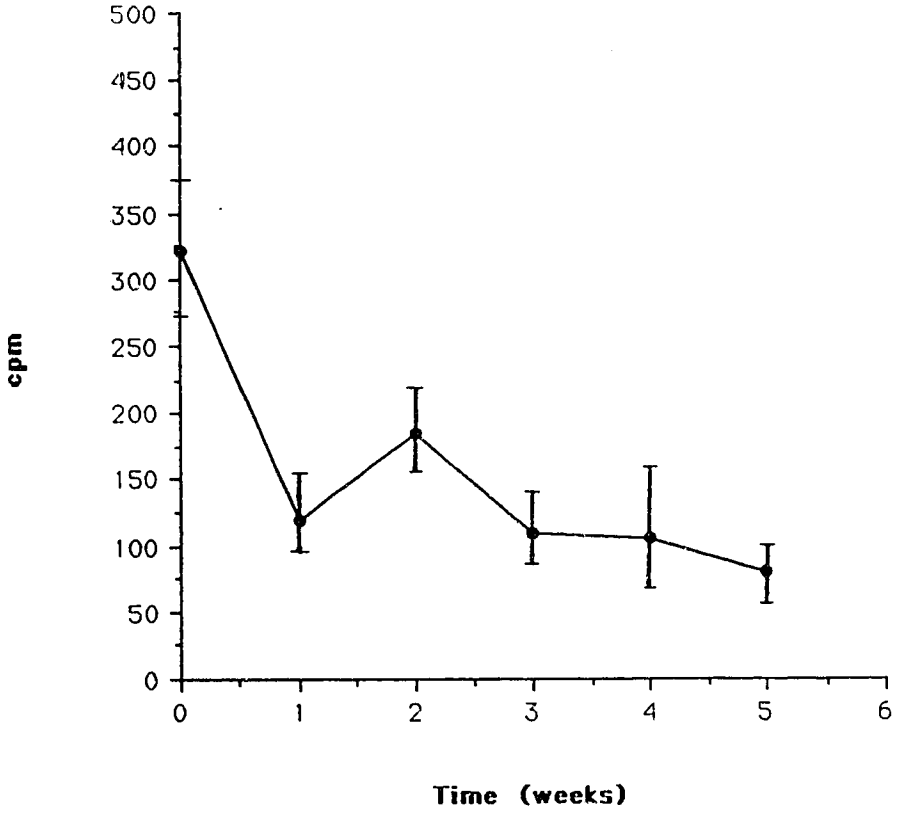

cpm percentage
120
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Time(hours)

Figure 3.2.

The half life of DHBV cccDNA in congenitally infected newly hatched ducklings. Each duckling was treated with 20 mg/kg of ddDAPR i.m. bid from birth. The cccDNA was extracted from the livers. An aliquot of 10 µl of DNA sample was evaluated by dot hybridization. After hybridization, the dots were cut for liquid scintillation counting. The amount of cccDNA was expressed as the radioactivity present in dots. Each point represents the mean value of 4 samples and the bars represent the range.


500
450
400
350
300
250
200
150
100
50
0
cpm
0
1
2
3
4
5
6
Time (weeks)

after 2 weeks of treatment. The poor response of newborn ducklings to ddDAPR treatment has been confirmed by Dr. Mang Ma, a resident in Internal Medicine currently investigating this observation in our laboratory. These observations led me to repeat these studies in 5-week-old animals. For these studies I want to acknowledge the assistance of Roopa Viraraghavan, a medical student who spent a summer in our laboratory. The study in 5-week-old ducklings showed a much more rapid decrease in the levels of cccDNA in liver tissue (Fig. 3.3) when compared with the decrease in cccDNA in animals treated from birth (Fig. 3.2). On the basis of the results of this study I have determined that the half life of cccDNA is approximately 4.2 days.

To confirm that the decrease of cccDNA as measured by the dot blots reflected a true decrease in cccDNA form, Southern blots were performed on there samples. The results of the Southern blot hybridization (one of three) are shown in figure 3.4 and a comparison of the mean of three Southern blot samples quantitated by scintillation counting is shown in figure 3.5. Unlike the results from dot hybridization, cccDNA tested by Southern blot analysis did not show significant decrease with ddDAPR treatment.

Figure 3.3.

The half life of DHBV cccDNA in congenitally infected 5-week-old ducklings measured by dot hybridization. Each duckling was treated with ddDAPR at 20 mg/kg i.m. q8h. DNA was extracted from livers of the animals. An aliquot of 10 µl DNA (5 mg of liver) was loaded onto nylon filter. After hybridization, the dots were cut for liquid scintillation counting. The relative amount of DNA is expressed as the amount of radioactivity present in the dots. Each point represents the mean value from 2 or 3 samples. The bar represent the range.

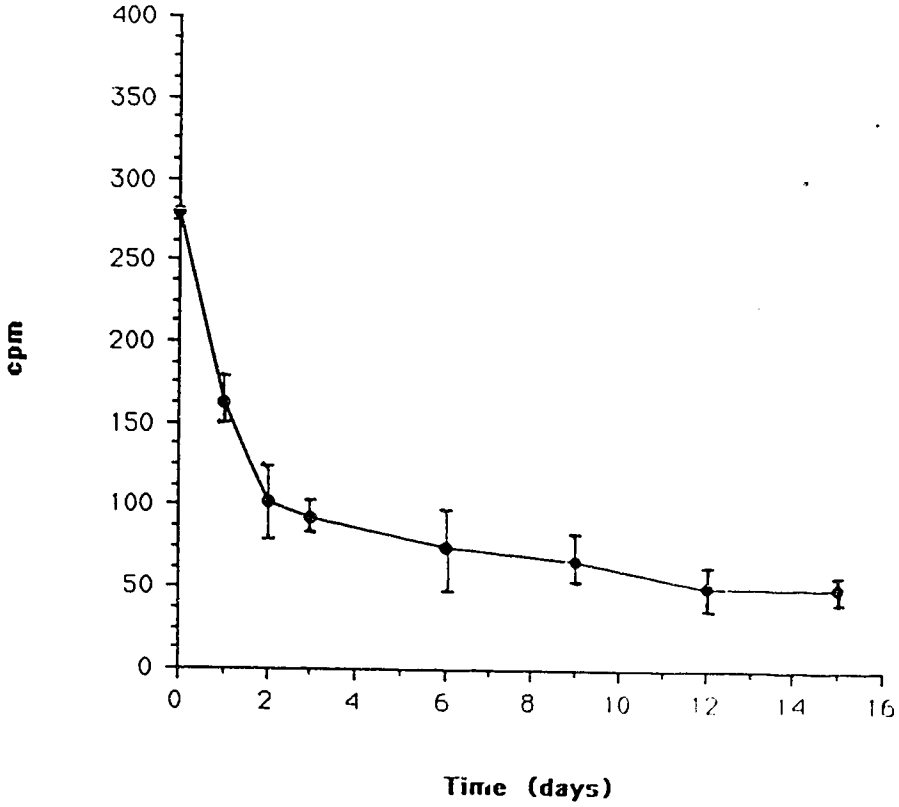
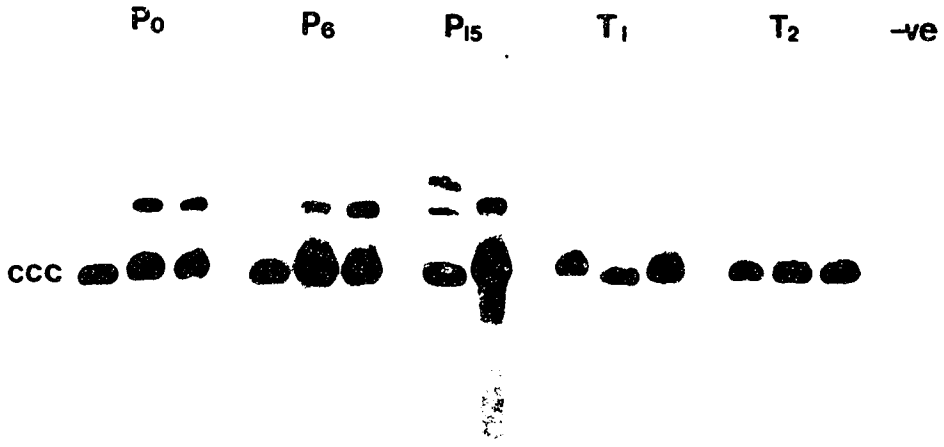

400
350
300
250
200
150
100
50
0
cpm
0
2
4
6
8
10
12
14
16
Time (days)

Figure 3.4.

Southern blot hybridization of cccDNA extracted from [illegible]y infected 5-week-old ducklings. DHBV-infected [illegible] were treated with placebo (20 mM Tris-HCl, pH 7.5 and [illegible]1, 2 ml/kg) or ddDAPR (20 mg/kg) i.m. q8h. Animals were sacrificed at the various time point (days) and cccDNA were extracted from the livers of animals. Each sample (20 µl [illegible] of liver tissues) was assayed by Southern blot hybridization. Letters P and T represent placebo treated and ddDAPR treated samples, respectively. Numbers besides the letter indicated the days of treatment. The position of cccDNA was indicated by "ccc" on the left side.


P0
P6
P15
T1
T2
-ve
ccc

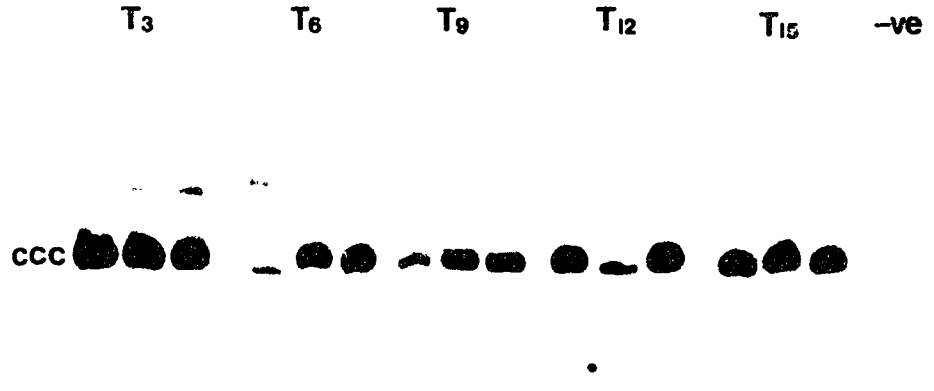
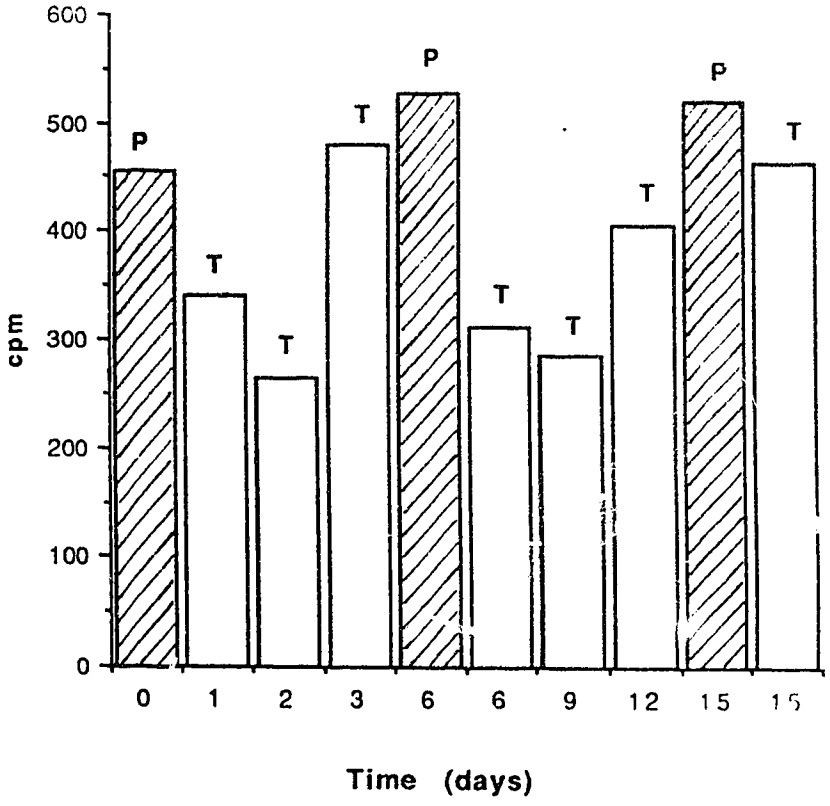

T3
T6
T9
T12
T15
-ve
ccc

Figure 3.5.

The amount of cccDNA in the livers of congenitally infected 5-week-old ducklings treated with ddDAPR as measured by Southern blot hybridization and liquid scintillation counting. Animals were treated and cccDNA was analysized as described in figure 3.3. The cccDNA bands detected in Southern blot hybridization (see figure 3.4) were cut out and counted by liquid scintillation. Each column represents the mean of two (for P15 only) or three (for all other columns) samples. The letters (P and T) represent placebo and drug treatment respectively and the numbers represent days of treatment.


600
500
400
300
200
100
0
cpm
P
T
T
T
P
T
T
T
P
T
0
1
2
3
6
6
9
12
15
15
Time (days)

3.3 The Effect of Duck Age on the Response to ddDAPR Treatment.

My previous studies had shown that treatment with ddDAPR at 10 mg/kg i.m. bid cleared the virus from serum by one week. The clearance of the virus from animals treated with 20 mg/kg i.m. bid was similar (incomplete clearance at 3 days, complete clearance by one week, based on dot hybridization). In a pilot study I found that newly hatched ducklings gave a much poorer response than animals that were 4 weeks old. To examine this more carefully, I treated 20 ducklings which were congenitally infected with DHBV. Ten ducklings were treated from birth with 20 mg/kg i.m. bid. Treatment in another 10 ducklings began at 4 weeks of age. As can be seen in Table 1, animals that were treated from hatching with ddDAPR showed a much slower clearance of the virus from their sera than did animals that were 4 weeks old when the treatment was initiated.

Table 3.1. The effect of age on the clearance of DHBV-DNA from the sera of ddDAPR-treated ducks.

Age of ducklings	Number of ducklings	Number of negative serum dot blots		
		1 week	2 weeks	3 weeks
newly hatched	10	0	5	5
4 weeks	10	10	N/A	N/A

N/A: Not Applicable.

DISCUSSION

The measurement of the half life of DHBV DNA in sera has been repeated in three separate studies with 5-week-old congenitally infected ducks. Two of them were performaed by treatment with ddDAPR at 20 mg/kg i.m. bid and the third experiment was done by treatment with ddDAPR at 20 mg/kg i.m. q8h. The disappearance of DHBV DNA from sera of treated ducks followed a similar pattern in each experiment with the half lives in the range of 11-14 hours. To the best of my knowledge this is the first estimate of the half life of a hepadnavirus in host sera. This study was made possible by the availability of a drug (ddDAPR) that inhibits viral reproduction. The disappearance of the virus from serum could be the result of several processes. Previous studies (Tuttleman, *et al.* 1986a, Wu, *et al.* 1990, Summers, *et al.*, 1990) have suggested that the cccDNA pool remains of constant size in hepatocyte. As cccDNA disappears from the pool, some of it is replenished by an intracellular pathway, however, some may also be contributed by extracellular reinfection. The clearance of DHBV from sera of ducks could be the result of virions leaving the blood stream to reinfect hepatocytes or other tissues. Although this is possible, the rapid disappearance of the virus makes it seem unlikely that all the virus could disappear by this route. The availability of a drug which prevents production of new virions has enabled me to study the removal of DHBV from the serum of its host.

My results can not necessarily be extrapolated to the human situation. Similar studies will have to be done in humans when an effective inhibitor of HBV replication is available and used in chronic carriers.

When I attempted to determine the half life of cccDNA in hepatocytes, I have considered that congenitally infected newly hatched ducklings would all have similar quantities of viral life cycle intermediates. Therefore, the use of newly hatched ducklings would reduce the sample error which I have seen in animals that are older. However, I did not know that newly hatched, congenitally infected ducklings would respond more slowly to treatment with ddDAPR. During the treatment period, I took sera from the treated newly hatched ducklings and assayed for viral DNA in these sera. The result showed clearly that the newly hatched ducklings were less sensitive to ddDAPR or cleared the virus slower than older ducks. However, I have repeated the experiment measuring the half life of cccDNA in hepatocytes in 5-week-old ducklings. I have assumed that treatment with ddDAPR at 20 mg/kg i.m. q8h will maintain the inhibitory effects of ddDAPR in ducks (unpublished results, Mang. Ma). Therefore I believe there is little opportunity to replenish the cccDNA pool by the intracellular route (Tuttlman, *et al.* 1986a, Wu, *et al.*, 1990, Summers, *et al.*, 1990). This allows me to try to estimate the half life of cccDNA. From the results of dot

hybridization, the amount of cccDNA decreased with the treatment by ddDAPR.

When I analysized these samples by Southern blot hybridization, the results show that cccDNA did not decrease much even after the 15 days of treatment with ddDAPR. I believe that the results from Southern blot hybridization are more reliable than the dot blot results.

The poor response of newly hatched ducklings to ddDAPR treatment could be the result of one or more differences in metabolism between newly hatched and older ducks. Firstly, the rate of deamination of ddDAPR could be slow in newly hatched animals and more efficient in 5-week-old ducks. Secondly, for it to be utilized in DNA synthesis, the conversion of ddDAPR to ddG must be followed by phosphorylation to ddGMP, ddGDP and ddGTP. The efficiency of this process may be considerably less in newly hatched ducklings than in older animals. Thirdly, the nucleoside pools may be quite different in new hatched ducklings than in older animals. If the dGTP pool was higher in newly hatched ducklings, then the effect of a given concentration of ddGTP would be much less. Fourthly, the newly hatched duckling may have a less effective immune system than a 5-week-old duckling. If rapid clearance of the virus from serum is a result of both antiviral and immune effects, then newly hatched ducklings would be expected to respond slowly given one or more of the above possibilities.

CHAPTER 4

STUDIES ON THE MECHANISM OF ACTION OF PURINE 2',3'-DIDEOXYNUCLEOSIDES

INTRODUCTION

Our observation that purine 2',3'-dideoxynucleosides, especially 2',3'-dideoxyguanosine analogues, effectively inhibit DHBV replication *in vitro* and *in vivo* invites several interesting questions. What is the mechanism of action of 2',3'-dideoxyguanosine analogues? Furthermore, is the mechanism of action applicable only to DHBV or does it also apply to other hepadnaviruses including HBV? Will the mechanism of action be specific enough to inhibit viral replication without seriously disrupting the metabolism of the host cell?

The chemical structure of 2',3'-dideoxynucleosides (Fig.2.1) is very similar to that of deoxynucleosides except that the 3'-OH on the deoxyribose is replaced by 3'-H in dideoxynucleosides. Therefore, if the 2',3'-dideoxynucleosides are metabolized in the cell, they may be incorporated into the elongating DNA and block the formation of the phosphodiester bond with the next nucleotide. The elongation of the DNA will be stopped. In this chapter, I examine the specificity of purine 2',3'-dideoxynucleoside

inhibition of DHBV replication. My work focused on a comparison between a purine 2',3'-dideoxynucleoside (ddGTP *in vitro* and ddDAPR *in vivo*) and a pyrimidine 2',3'-dideoxynucleoside (ddCTP *in vitro* and ddC *in vivo*) with respect to: (a) their competive and chain terminative effects on the DNA polymerase and reverse transcriptase activities of isolated replicative core particles; and (b) their effects on the synthesis of viral nucleic acid in replicative core particles isolated from treated animals. In addition, I attempted to demonstrate the binding of purine 2',3'-dideoxynucleotides to the terminal protein.

MATERIALS AND METHODS

Chemicals. 2',3'-Dideoxyguanosine 5'-triphosphate (ddGTP), 2',3'-dideoxycytidine 5'-triphosphate (ddCTP) and the deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP) were purchased from Pharmacia Canada Inc. ddDAPR and ddC were synthesized by Raylo Chemicals, Edmonton, Canada. [α^{32}P]dCTP and [α^{32}P]dGTP were purchased from Amersham Canada Ltd. Dideoxyadenosine 5'-[α-thio] triphosphate ^{35}S (α^{35}S-ddATP) was purchased from DuPont Canada Inc.

Ducks and Treatment. Congenitally infected 4-week-old ducks were treated for two weeks with placebo (20 mM Tris-HCl, pH 7.5 and 0.72% of NaCl, 2 ml/kg), ddDAPR (10 mg/kg) or ddC (10 mg/kg) by intramuscular injection twice daily. The dose of 10 mg/kg i.m. bid was selected on the basis of rapid clearance of DHBV DNA from sera by ddDAPR treatment (Fig. 2.3 and Fig. 2.4). ddC, which was not active at this dose, was used as negative control (Fig. 2.4). After treatment was complete, the animals were anesthetized with intravenous pentobarbital sodium, the livers were removed, frozen in liquid nitrogen and stored at -70° C until use for the preparation of replicative core particles.

Preparation of Replicative Core Particles. DHBV replicative core particles were prepared by two methods. The first method has been described by Summers and Mason (1982) and was used here with minor modifications. Briefly, 1 g of

liver was cut into small pieces and homogenized in 30 ml of extraction buffer (EB) containing 20 mM Tris-HCl pH 7.4, 7 mM $MgSO_4$, 50 mM NaCl, 0.1% 2-mercaptoethanol, 100 µg/ml bovine serum albumin and 0.25 M sucrose. The homogenized suspension was centrifuged at 12,000 rpm for 20 minutes at 4° C in a Beckman JA20 rotor. Five ml of the supernatant was layered onto 33 ml of a 15%-30% linear sucrose gradient in EB and centrifuged at 23,000 rpm for 7 hours at 4° C in a Beckman SW27 rotor. Fractions of the sucrose gradient were collected in 1.5 ml portions and assayed for DNA polymerase activity by endogenous labelling (see below). The fractions showing DNA polymerase activity were pooled and pelleted by centrifugation at 23,000 rpm for 15 hours at 4° C in a Beckman SW27 rotor. The pellets were suspended in 4 ml of EB buffer and treated with DNase (1 µg/ml) at 37° C for 10 minutes. After the addition of EDTA to produce a solution of 20 mM, the suspension was further treated with RNase A (0.1 µg/ml) for another 10 minutes. The suspension was centrifuged for 5 minutes in a clinical centrifuge. Two ml of supernatant were then layered onto 11 ml of a 15-30% linear sucrose gradient made in EB buffer and centrifuged at 23,000 rpm for 15 hours at 4° C in a Beckman SW40 rotor. The pellet containing the replicative cores was carefully collected as previously described to prevent mixing with supernatant (Summers and Mason, 1982). The replicative cores purified from 10 g of liver tissue were suspended in 2 ml of EB buffer for extraction of DNA and pregenomic RNA or in 2 ml

of 10 mM Tris HCl, pH 7.5 for testing inhibition of enzymatic activities. The cores were stored at -20° C in 0.5 ml aliquots until used for nucleic acid extraction or enzymatic assays as described below.

The second method used for the preparation of replicative core particles was a miror modification of the method described by Bartenschlager and Schaller (1988). Approximately 20 g of liver tissue was sliced and 50 ml of TPT buffer (10 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 1 mM PMSF) were added prior to homogenization in a Dounce homogenizer. The homogenized solution was centrifuged in a Beckman JA20 rotor at 12,000 rpm at 4° C for 20 minutes. The supernatant was loaded onto a step gradient containing 6 ml each of 60%, 30% and 15% sucrose in TPT. After centrifugation in a Beckman SW28 rotor at 25,000 rpm at 4° C for 4 hours, gradient fractions were collected from the bottom of the tube and fractions containing the viral replicative complexes were detected using dot hybridization for DHBV DNA. Positive fractions were pooled and centrifuged at 23,000 rpm at 4° C for 15 hours. The pellet was suspended in EB buffer and treated with DNase I and RNase A as described above. After treatment, the suspension was loaded onto a linear gradient of 15%-60% sucrose in TPT buffer and centrifuged in a Beckman SW40 rotor at 23,000 rpm at 4° C for 16 hours. Fractions were collected from the bottom of the tube and assayed for DHBV by dot hybridization. These

fractions were utilized for endogenous labelling of DHBV DNA or Southern blot analysis.

Extraction of Viral DNA from Replicative Core Particles. Replicative core aliquots of 0.5 ml were incubated with proteinase K (0.5 mg/ml) in the presence of 0.5% SDS at 37° C for 30 minutes. The lysate was extracted twice with an equal volume of phenol and then with an equal volume of chloroform. One tenth volume of 3.0 M NaOAc was added to the aqueous phase and the nucleic acids were precipitated in 2.5 volumes of ethanol at -70° C for 30 minutes. Nucleic acid samples were recovered by centrifugation in an Eppendorf centrifuge for 15 minutes at 4° C. The pellet was washed once with cold 70% ethanol and dissolved in 20 µl of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The nucleic acids were treated with RNase H (RNase H 4 units/20 µl, Bethesda Research Laboratories) at 37° C for 30 minutes, followed by a phenol and a chloroform extraction. The viral DNA was precipitated in 0.3 M NaOAc, pH 7.0 and 2 volumes of ethanol at -70° C for 30 minutes. The DNA was pelleted in an Eppendorf centrifuge for 15 minutes at 4° C. The pellet was dissolved in 80 µl of TE buffer and 20 µl was used for Southern blot analysis.

Extraction of Pregenomic RNA from Replicative Core Particles. The total nucleic acids were extracted from replicative core particles as described above. After precipitation, the nucleic acids were dissolved in 40 mM

Tris-HCl (pH 7.9), 10 mM NaCl and 6 mM $MgCl_2$. The solution was heated at 100° C for 1 minute and immediately cooled on ice in order to separate any DNA:RNA hybrids. After a quick spin in an Eppendorf centrifuge, DNase (RNase-free DNase 4 u/20 µl, Promega) was added to the solution which was incubated at 37° C for 30 minutes followed by phenol and chloroform extractions. Viral RNA was precipitated in 0.3 M NaOAc pH7.0 with 2.5 volumes of ethanol at -70° C for 30 minutes. The RNA in this solution was divided into 4 equal aliquots and stored in 0.3 M NaOAc and 2.5 volumes of ethanol until it was used for Northern blot studies. On the day that the Northern blot was to be performed, the RNA was pelleted in an Eppendorf centrifuge for 15 minutes at 4° C. The RNA pellet was dissolved in 4.7 µl of H_2O and treated as described below (see Northern blot hybridization).

Endogenous Labelling. Aliquots of 15 µl of the sucrose gradient fractions were mixed with an equal volume of 3 x polymerase buffer (1 x polymerase buffer contains 50 mM Tris-HCl pH8.0, 50 mM NaCl, 20 mM $MgCl_2$, 0.1% NP-40, 50 µM dATP, 50 µM dGTP, and 50 µM dTTP) at room temperature for 10 minutes. [α-^{32}P] labelled dCTP (2 µCi/15 µl) was added to this mixture which was incubated at 37° C for 1 hour. The DNA was precipitated from the reaction mixture with 10% trichloroacetic acid (TCA) on ice for 10 minutes. The precipitate was collected by filtration of the solution through a GF/C glass-fiber disc and washed 3 times with 5 ml

of cold 10% TCA followed by 3 washes with 3 ml of ethanol. The GF/C glass-fibers filters were dried and counted in a liquid scintillation counter.

Inhibition of Replicative Core DNA Polymerase and Reverse Transcriptase Activities by Dideoxyucleotide Analogues. Replicative core particles in 10 mM Tris-HCl pH 7.5 (4 µl) were mixed with 4 µl of 4x polymerase buffer at room temperature for 10 minutes. The inhibitors (ddGTP or ddCTP) in 4 µl and [α-^{32}P] labelled dGTP or dCTP in 4 µl were added to the replicative core mixture and incubated at 37° C. Final concentrations for the measurement of the effect of ddGTP were as follows: 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 mM $MgCl_2$, 0.1% NP-40, 50 µM of dATP, dCTP, and dTTP and 0.1 µM of [α-^{32}P]dGTP with various concentrations of ddGTP. Similarly the effect of ddCTP was measured in the polymerase buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 20 mM $MgCl_2$, 0.1% NP-40, 50 µM of dATP, dGTP, and dTTP and 0.1 µM of [α-^{32}P]dCTP with various concentrations of ddCTP. When core particles were assayed for reverse transcriptase activity, actinomycin D was added to the polymerase buffer at 100 µg ml. The reaction was stopped after 2 minutes by the addition of 4 µl of a mixture containing 100 mM EDTA and 0.5% SDS. The replicative core particles were incubated at 37° C for another 30 minutes, followed by precipitation in 5 ml of 10% TCA. The inhibitory effects of ddGTP and ddCTP were measured by calculating the decrease in the incorporation of

[α-^{32}P] labelled deoxynucleotides into the TCA precipitated nucleic acids. In these experiments, I followed the procedure for DNA polymerase assay described by Bartenschlager and Schaller (1988). I also measured the incorporation of [α-^{32}P] labelled deoxynucleotides into TCA precipitated nucleic acids over a short time period with the replicative core particles I prepared. The time (2 minutes) selected for the inhibition test was well below the time (10 minutes) required for the reaction to plateau under the conditions I have used. The inhibitory effect is best determined during the phase of rapid incorporation of radiolabelled deoxynucleotides

Electrophoresis of the Products of Endogenous Labelling. The reaction mixture for endogenous labelling was heated to 60° C for 20 minutes after the addition of SDS (2%) and ß-mercaptoethanol (2%). One µg of tRNA was added to the mixture and the nucleic acids were precipitated with 0.3 M NaOAc and 2 volumes of ethanol at -70° C. The precipitate was pelleted by centrifugation in an Eppendorf centrifuge at 4° C for 15 minutes. After the sample was dried, the pellet was dissolved in 10 µl of TE buffer and electrophoresed on a 1.5% SDS-agarose gel with TAE buffer containing 0.1% SDS. After electrophoresis, the gel was rinsed with TAE buffer for 30 minutes with gentle agitation, dried overnight and exposed to X-ray film at room temperature for approximately 2 hours.

Northern Blot Hybridization. A sample of viral RNA in H_2O (4.7 µl) was added to 14 µl of solution containing 10 µl of formamide, 2 µl of formaldehyde and 2 µl of 10x MOPS (10x MOPS contains 200 mM MOPS, 50 mM NaOAc and 10 mM EDTA, pH 7.0). The RNA solution was heated to 65° C for 10 minutes and the liquid condensed inside the tube was brought down by a short centrifugation in an Eppendorf centrifuge. The RNA was electrophoresed in a 1% agarose gel with running buffer containing 1x MOPS and 1 M formaldehyde (37% formaldehyde is 12.3 M). After electrophoresis, the gel was soaked in 20x SSC at room temperature for 30 minutes with gentle shaking. The RNA fragments were transferred to a nylon membrane with 20x SSC and fixed onto the membrane by baking at 80° C for 2 hours. Prehybridization was performed at 42° C overnight in 5x PIPES (20x PIPES contains 3 M NaCl, 0.1 M PIPES and 0.1 M EDTA), 50% formamide, 5x Denhardt's solution, 0.2% SDS, and 100 µg/ml of denatured salmon sperm DNA. Hybridization was done in the presence of an [α-^{32}P] labelled DHBV DNA probe under the same conditions as the prehybridization. The membrane was washed 4 times in 0.5x SSC and 0.1% SDS at 65° C for 30 minutes each. Following this, the membrane was washed in 0.5x SSC at room temperature for 30 minutes, wrapped in Saran wrap and exposed to X-ray film as described previously (see Chapter 2, dot hybridization).

Preparation of Nuclear and Cytoplasmic Extracts for Terminal Protein Binding to [α^{35}S]ddATP. An

attempt to demonstrate the binding of [α^{35}S]ddATP to the terminal protein was based on studies done with adenovirus by Challberg, *et al.* (1980). Primary hepatocyte cultures were prepared in 60-mm tissue culture plates (about 2.5 x 10^6 cells/plate) from DHBV infected ducks. Cells were cultured at 37° C for 8 days and collected by scraping the cell with a rubber pliceman in ice cold buffer containing 20 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 50 mM NaCl and 0.25 M sucrose. The cell suspension was centrifuged in a Beckman JA20 rotor at 3000 rpm at 4° C for 5 minutes. The supernatant was removed and the cell pellet was suspended in the same buffer (1 x 10^8 cells/2.5 ml) with 0.1% 2-mercaptoethanol and 700U/ml RNase inhibitor (RNasin). The cell suspension was homogenized by 10 strokes in a Dounce homogenizer and centrifuged in Beckman JA20 rotor at 4000 rpm at 4° C for 5 minutes to pellet the nuclei. The supernatant, containing the cytoplasmic fraction, was clarified by centrifugation at 12,000 rpm for 20 minutes. This cytoplasmic extract was stored at -20° C in 200 µl aliquots. The nuclear pellet was suspended in 1.25 ml of 50 mM Tris-HCl pH 7.5, 100 mM NaCl and 10% sucrose and kept on ice for 1 hour, before being centrifuged in an Eppendorf centrifuge at 4° C for 20 minutes. The supernatant was stored at -20° C in 100 µl aliquots. The protein concentrations of the cytoplasmic extract and nuclear extract were determined by a modified Lowry method (Markwell, *et al.*, 1978).

Binding Assay. The binding of [α-^{35}S]ddATP to viral protein was carried out in a 100-µl reaction mixture containing 140 µg of cytoplasmic extract, 30 µg of nuclear extract, 100 mM ATP, 4 µl of [α-^{35}S]ddATP (12.4 mCi/ml) and 25 units of RNase inhibitor (Amersham Canada Ltd) at 37° C for 1 hour. The reaction was stopped by the addition of EDTA to 20 mM followed by precipitation in 10% TCA on ice for 10 minutes. The pellet was washed with cold acetone once and dried. The sample was dissolved in 80 µl of sample buffer (62.5 mM Tris-H_3PO_4 pH 6.8, 2% SDS, 0.2% DTT, 0.1% EDTA and 10% glycerol), heated to 100° C for 2-5 minutes and electrophoresed on a 10% SDS-PAGE gel. The gel was soaked in 1 M sodium salicylate for 30 minutes at room temperature with gentle agitation, dried and exposed to X-ray film with an enhancer screen at -70° C.

Preparation of DHBV Virions from Sera of DHBV Infected Ducks. The sera of DHBV infected ducks were centrifuged at 5,000 rpm at 4° C for 10 minutes to remove debris, layered over 20 ml of a 10%-20% sucrose gradient in 0.15 M NaCl and 20 mM Tris HCl, pH7.4 and centrifuged in a Beckman SW27 rotor at 23,000 rpm at 4° C for 16 hours. The pellet containing the virus was suspended in TE buffer and sonicated with a needle sonicator for one minute at 4° C. The virions were treated with nuclease (2 units/ml, *Staphylococcus aureus*) in the presence of 7 mM $CaCl_2$ at 37° C for 30 minutes after which the treatment was stopped by

addition of EGTA to 10 mM. The virions were layered on 4 ml of a 15%-30% sucrose gradient and centrifuged in a Beckman SW60 rotor at 50,000 rpm at 4° C for 3 hours. The pellet was again suspended in TE buffer by sonication for one minute.

Preparation of Terminal Protein-DNA Complex (TPDC) from DHBV Virions. The TPDC was prepared using the method previously described by Molnar-Kimber, *et al.* (1983) with minor modifications. A DHBV virion suspension harvested from 300 ml of serum from DHBV positive ducks was adjusted with SDS to 2% and ß-mercaptoethanol to 2% and incubated at 60° C for 20 minutes to disrupt the virions. The suspension was layered onto 4 ml of a 5% -20% sucrose gradient prepared in 10 mM Tris-HCl, pH7.4, 10 mM EDTA, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged in a Beckman SW60 rotor at 50,000 rpm at 4° C for 4.3 hours. Fractions (200 µl) were collected from the bottom of the tube. DHBV-DNA positive fractions, as identified by dot hybridization, were pooled and precipitated with 0.3 M NaOAc and 2 volumes of cold ethanol. The TPDC was pelleted by centrifugation in a Beckman JA20 rotor at 12,000 rpm at 4° C for 30 minutes. The pellet was dissolved in 10 mM Tris-HCl, pH7.5, 10 mM EDTA and 0.1% SDS by heating to 68° C in a water bath. The SDS was then removed by washing the solution 4 times with 1 ml of TE buffer in a centricon-30 microconcentrator (Amicon Canada Ltd) in a Beckman JA20 rotor at 7,000 rpm at 20° C for 20 minutes. The TPDC solution was

adjusted to 0.3 M NaCl and loaded onto a benzoylated naphthoylated DEAE (BND)-cellulose column (Serva Feinbiochemica GMBH.& Co) by passing the solution though the column 3 times (Heuson, 1978). The column was washed 3 times with 3 column-volumes of 0.3 M NaCl, 10 mM Tris-HCl pH 7.4, and 10 mM EDTA. Double stranded DNA was eluted with 3 column-volumes of 1 M NaCl, 10 mM Tris HCl, pH 7.4 and 10 mM EDTA. Single stranded DNA was eluted with 3 column-volumes of 1 M NaCl, 2% caffeine, 10 mM Tris HCl, pH 7.4 and 10 mM EDTA. TPDC was eluted with 3 column-volumes of 1 M urea, 1% SDS, 10 mM Tris HCl, pH 7.4 and 10 mM EDTA. Each type of effluent was collected in 0.5 ml fractions. Complete elution of each DNA species was confirmed by dot hybridization showing the absence of DHBV-DNA in the later effluents for each step. The effluent containing TPDC was washed 5 times with 10 mM Tris-HCl pH 7.5 and 0.1 mM EDTA in a centricon-30 microconcentrator. The amount of TPDC obtained was estimated to be about 8×10^{-2} pico moles.(170 ng DNA) by dot hybridization and comparison with known amounts of DHBV-DNA in the plasmid. The presence of terminal protein linked to the DHBV-DNA molecule was confirmed by a change in the mobility of DHBV-DNA on 1.5% agarose-SDS gel after digestion with proteinase K (Molnar-Kimber, *et al.* 1983).

Iodination of TPDC. Iodination of the protein in the TPDC was done by two methods. The first method was iodination with Iodo-Gen (1,3,4,6-tetrachloro-3a, 6a-diphenyl

glycouril, Pierce Chemical Co.). Twenty µl of Iodo-Gen (2 mg/ml in chloroform) were added to an acid cleaned glass tube (12 x 75 mm). The tube was slowly vortexed and the chloroform was evaporated under a gentle stream of nitrogen. The Iodo-Gen coated tubes were stored at 4° C in the dark. The reaction was carried out by addition of TPDC (20 ng of DNA/40 µl) and [^{125}I]Na (8 mBq/2 µl) to the tube which was rotated on ice for 5 minutes. After passing the reaction mixture though a glass-wool-plugged Pasteur pipet, the reaction was stopped by passing 40 µl of cysteine (1 mg/ml in PBS) through the Pasteur pipet.

A second method for iodination of TPDC used the Bolton and Hunter reagent (N-succinimidyl 3- [4-hydroxy-3, 5-[^{125}I] di-iodo-phenyl] propionate, Amersham Canada Ltd). TPDC (20 ng of DNA/40 µl) was coprecipitated with 2 µg of tRNA. The nucleic acids were pelleted by centrifugation in an Eppendorf centrifuge for 15 minutes. The pellet was dried and dissolved in 10 µl of 0.1 M borate buffer pH 8.5 (prepared by the addition of 0.1 M Na borate to 0.1 M boric acid until the pH reached 8.5). Bolton and Hunter reagent (0.5 mCi) in organic solvent was evaporated to dryness as described in the instruction sheet and the tube was placed on ice immediately. The TPDC in borate buffer was transferred to this tube and the reaction was allowed to proceed on ice for 15 minutes with gentle agitation.

After labelling the TPDC by either method, the sample was transferred to a millipore disc filter with pore size 0.05 µm (millipore VMWP0.05, Millipore Corp. Bedford, Mass.) and dialyzed against 10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA overnight. The TPDC samples were washed 4 times in a centricon-30 microconcentrator, with 1 ml of 10 mM Tris-HCl, pH 7.5. The sample containing about 4 x 10^6 cpm in a volume of 2 µl was subjected to enzymatic digestion as described below.

Enzymatic Digestion of [^{125}I] Labelled TPDC. [^{125}I] Labelled TPDC was digested with DNase I (5 units/10 µl, Sigma Chem. Co.) in 50 mM Tris-HCl pH 8.0 and 10 mM $MgCl_2$, or with Micrococcal endonuclease (1 unit/ 10 µl, Sigma Chem. Co.) in 50 mM Tris-HCl, pH 8.8 and 7 mM $CaCl_2$, or proteinase K (5 µg/10 µl, Sigma Chem. Co.) in 50 mM Tris-HCl, pH 8.0 and 0.5% SDS. All digestion mixtures were incubated at 37° C for 2 hours. After digestion, the samples were analyzed by electrophoresis on a SDS-Urea-PAGE gel (SDS-UREA gel, Pharmacia Canada Inc.) followed by exposure to X-ray film for 2-4 hours at room temperature.

RESULTS

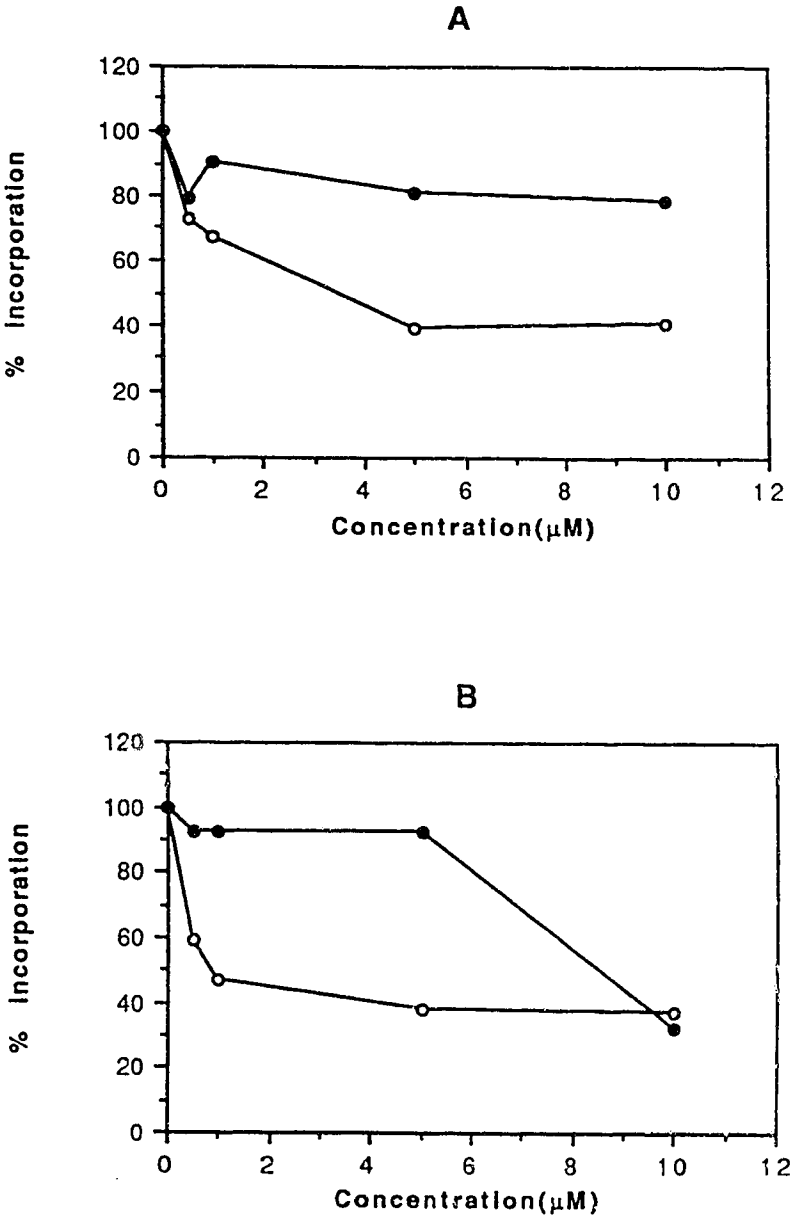
4.1 Effects of ddGTP and ddCTP on DHBV DNA Polymerase and Reverse Transcriptase Activities.

The competition and chain termination effects of ddCTP and ddGTP on the viral DNA polymerase or reverse transcriptase activities were studied utilizing endogenous labelling in the replicative core particles. The polymerase of DHBV contains both DNA polymerase and reverse transcriptase activities. Therefore the products of ^{32}P-dNTP incorporation represent a composite of both activities. The addition of actinomycin D to the reaction mixture blocks DNA polymerase activity and allow us to examine the effects of ddGTP or ddCTP on reverse transcriptase activity alone.

To examine these effects of ddGTP on enzymatic activity, the reaction mixture was adjusted to contain [α-^{32}P]dGTP at a relatively low concentration (0.1 µM) and ddGTP at increasing concentrations of 0.5 µM to 10 µM. The result is shown in figure 4.1 A. ddGTP was not an effective competitor of dGTP binding to the DHBV polymerase (DNA polymerase and reverse transcriptase) since ddGTP at concentrations 100-fold higher than the concentration of dGTP (10 µM ddGTP vs 0.1 µM [α-^{32}P]dGTP), decreased the incorporation of [α-^{32}P]dGTP into viral DNA by only 20%. When actinomycin D was added to the reaction mixture at 100 µg/ml, the incorporation of

Figure 4.1.

Effect of ddGTP or ddCTP on the incorporation of the analogous nucleotide into DHBV-DNA by polymerase and reverse transcriptase activities of DHBV. Panel A: Inhibition of nucleotide incorporation into DHBV DNA by DNA polymerase (•-•) and reverse transcriptase (o-o) by ddGTP. DNA polymerase activity was measured by the incorporation of [α-^{32}P] labelled dGTP into TCA precipitative material. Reverse transcriptase was measured by incorporation of [α-^{32}P] labelled dGTP in the presence of actinomycin D (100 µg/ml). The results are expressed as percentages of radioactivity incorporated relative to the amount incorporated in the absence of chain terminators (100%). Each datum point represents the mean of three individual experiments. Panel B: Experimental conditions were similar to those described for panel A except that incorporation into TCA precipitative material was measured after the incorporation of [α-^{32}P] labelled dCTP, and ddCTP was used in place of ddGTP.


A
% Incorporation
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration(μM)
B
% Incorporation
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration(μM)

[α-^{32}P]dGTP into the minus strand of DNA was decreased by about 10% at high concentration s of ddGTP.

The competition and chain termination effects of ddCTP on DHBV-DNA polymerase was examined in experiments that were similar to those used for ddGTP (Figure 4.1. B). The reactions were carried out in the presence of 0.1 µM of [α-^{32}P] dCTP with varying concentrations of ddCTP. Like ddGTP, ddCTP was a relatively weak chain terminator for this reaction (a composite of DNA polymerase and reverse transcriptase). In the presence of actinomycin D, ddCTP at a concentration 10 times higher than that of [α-^{32}P] dCTP resulted in about a 60% decrease in the incorporation of [α-^{32}P] dCTP. Under the conditions used in these experiments, the 50% inhibition nucleotide incorporation was 3.2 µM and 0.9 µM for ddGTP and ddCTP respectively.

4.2 A Comparison of the Effect of ddDAPR or ddC Treatment on Viral Nucleic Acids in Replicative Core Particles.

I have examined viral DNA and pregenomic RNA in replicative core particles isolated from the livers of ducks treated with 2',3'-dideoxynucleosides in order to test the effects of these compounds on viral nucleic acids in these particles. Four week old animals were treated for two weeks with placebo (2ml/kg), ddDAPR (10 mg/kg) or ddC (10 mg/kg) given by intramuscular injection, twice daily. The

replicative core particles were isolated from the livers of treated ducks using the method described by Summers and Mason (1982). Viral DNA and pregenomic RNA were extracted from the replicative core particles. The results of Southern blot hybridization are shown in figure 4.2. Viral DNA in the replicative core particles of placebo-treated animals contains two major forms of DHBV DNA, namely linear and relaxed circular (RC) forms (Fig. 4.2, lane C). Treatment with ddC (Fig. 4.2, lane B) resulted in a profile of viral DNA that changed very little indicating little or no inhibitory effect of ddC on the viral DNA in the replicative core particles. However, treatment with ddDAPR dramatically decreased viral DNA in the replicative core particles (Fig. 4.2, lane A). With longer exposure a weak band could be detected at the RC position (data not shown). This suggested that ddDAPR strongly inhibited DHBV replication at the level of DNA synthesis in the replicative core particles. The nearly complete inhibition of DNA synthesis suggests thatsynthesis of both strands of viral DNA was affected by ddDAPR treatment.

The results of Northern blot hybridization are shown in figure 4.3. Pregenomic RNA in the sample from placebo treated ducks (Fig. 4.3, lane C) shows 2 major bands in the 3.4-3.5 kb range. There is also a smear below 3.4 kb. This probably represents the status of the pregenomic RNA in the replicative core particles during the DNA synthesis, although

Figure 4.2.

Southern blot hybridization of DHBV DNA from replicating core particles isolated from livers of drug-treated and placebo-treated ducks. Animals were treated for 2 weeks and DNA was extracted from replicating cores isolated from liver tissue. Electrophoresis was performed in 1% agarose in TBE buff . Lanes A, B, and C represent the DNA from ddDAPR, ddC and placebo treated ducks, respectively. Positions of the markers are indicated on the left, with molecular weight being indicated in kilobases. The letters on the right identify relaxed circular (RC) and linear (L) forms of DHBV DNA.

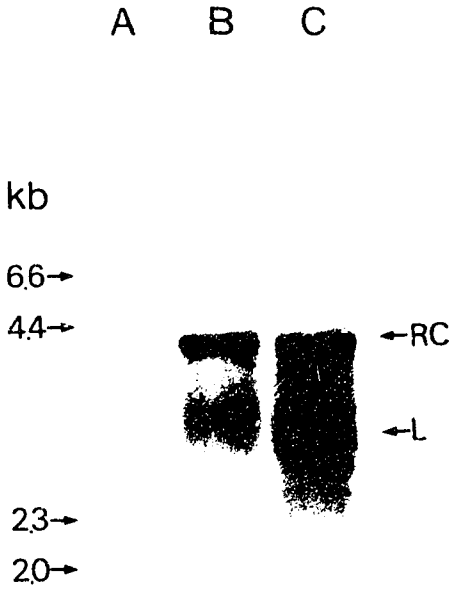
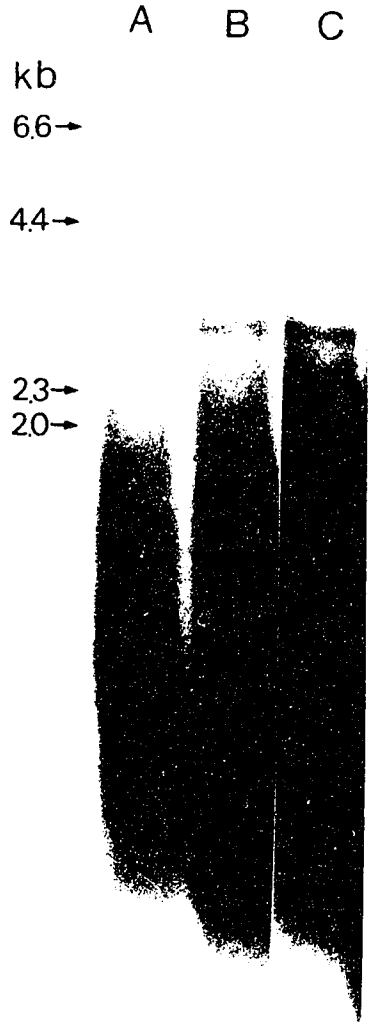

A
B
C
kb
6.6
4.4
2.3
2.0
RC
L

Figure 4.3.

Northern blot hybridization of DHBV RNA from replicating core particles isolated from drug-treated and placebo-treated duck livers. Animals were treated for 2 weeks prior to sacrifice. Electrophoresis was performed in a 1% agarose gel containing 1 M formaldehyde. Lanes A, B and C represent the samples obtained from ducks treated with ddDAPR, ddC and placebo, respectively. The position of the molecular weight markers are indicated in kilobases on the left.


A
B
C
kb
6.6
4.4
2.3
2.0

some RNA breakdown may also have occurred during its preparation. This result was consistently obtained in four experiments. Pregenomic RNA in the replicative cores isolated from ddC treated animals (Fig. 4.3, lane B) was almost identical to that from placebo treated animals, which indicates that treatment with ddC had little effect on synthesis of pregenomic RNA. In contrast, treatment with ddDAPR decreased the amount of pregenomic RNA in replicative core particles (Fig. 4.3, lane A). However, the reduction in RNA was not as great as the reduction in viral DNA.

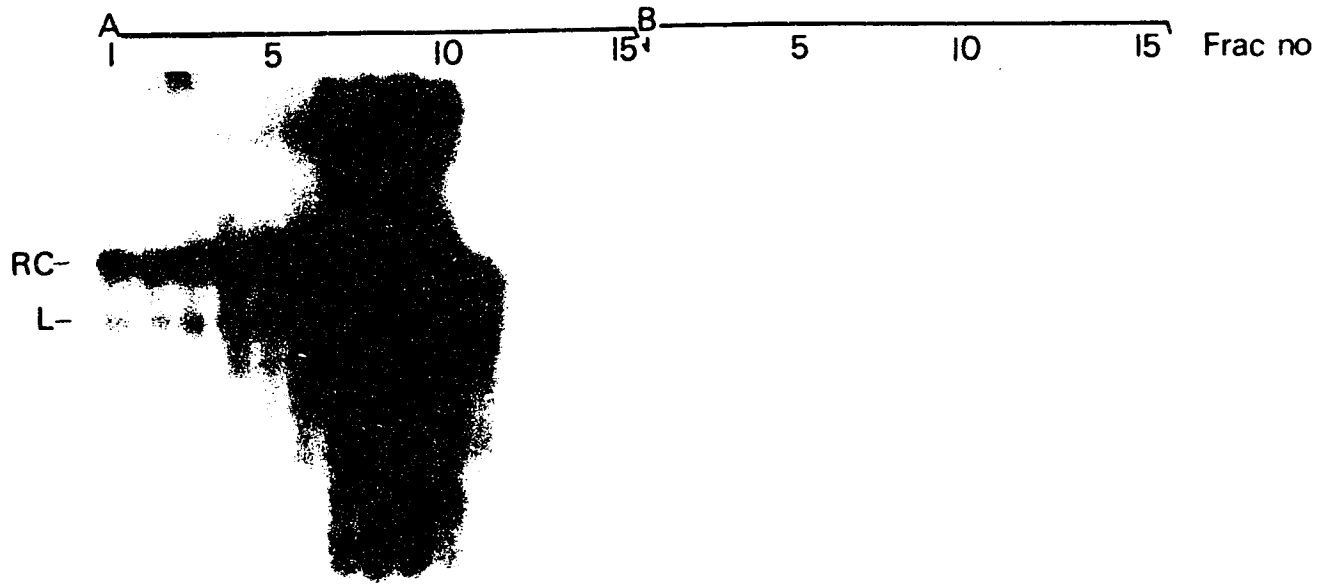
In summary, viral DNA synthesis in the replicative complex was almost completely inhibited by treatment with ddDAPR. ddDAPR treatment also decreased the level of pregenomic RNA, but to a much lesser extent, which suggests that this effect may be indirect. Treatment with ddC did not show any inhibitory effect on the accumulation of core particles containing viral DNA or pregenomic RNA.

Another set of experiments was done to examine more specifically the inhibitory effect of ddDAPR on the viral nucleic acids after 2 weeks of treatment with ddDAPR at 10 mg/kg, i.m.,bid. In this study, the replicative core particles were isolated from sucrose gradient fractions by the method of Bartenschlager and schaller (1988), which gave a little better endogenous labelling than the method of Summers and Mason (1982). I examined the ability of replicative core particles to incorporate radiolabelled

deoxynucleotides into viral DNA by endogenous labelling. The core particles in each fraction (1 to 15) from a sucrose gradient were tested in a separate endogenous labelling reaction. The cores were then disrupted and the products of the labelling were electrophoresed on 1.5% agarose gels containing 0.1% SDS. The gels were dried and exposed to X ray film for autoradiography. In the preparations from placebo-treated animals, most of the label in the replicative core particle fractions numbered 1-5 was found in the RC band whereas fractions numbered 6-10 had labelled DNA of various forms including single stranded, linear and RC (Fig. 4.4, panel A). There was very little labelled product detectable after endogenous labelling reactions with the replicative core particles in similar sucrose gradient fractions taken from ddDAPR treated animals (Fig. 4.4, panel B). Only a small smear of single stranded DNA was seen in fractions numbered 7-8. This indicated that viral DNA elongation was blocked by treatment with ddDAPR. All of these animals were sacrificed 6 hours after receiving the last dose of ddDAPR. Studies have shown that the half life of the drug is approximately 30 min in duck blood (personal communication, T. Kitos). This fact, combined with the fact that the preparation of the replicative core involves centrifugation through two sucrose gradients, would imply that there should be no free ddDAPR in these preparations to inhibit reverse transcriptase or DNA polymerase during the endogenous labelling reaction.

Figure 4.4.

SDS-agarose gel electrophoresis followed by autoradiography of endogenously labelled viral DNA products. Numbers 1 to 15 across the top indicate the sucrose gradient fractions collected after centrifugation of samples from placebo (panel A) or ddDAPR treated (panel B) ducks. Each fraction was used in a separate reaction for endogenous labelling, following which the reaction mixtures were subjected to electrophoresis and autoradiography. Letters on the left identify relaxed circular (RC) and linear (L) forms of the viral DNA.


A
B
1
5
10
15
5
10
15
Frac no
RC–
L–

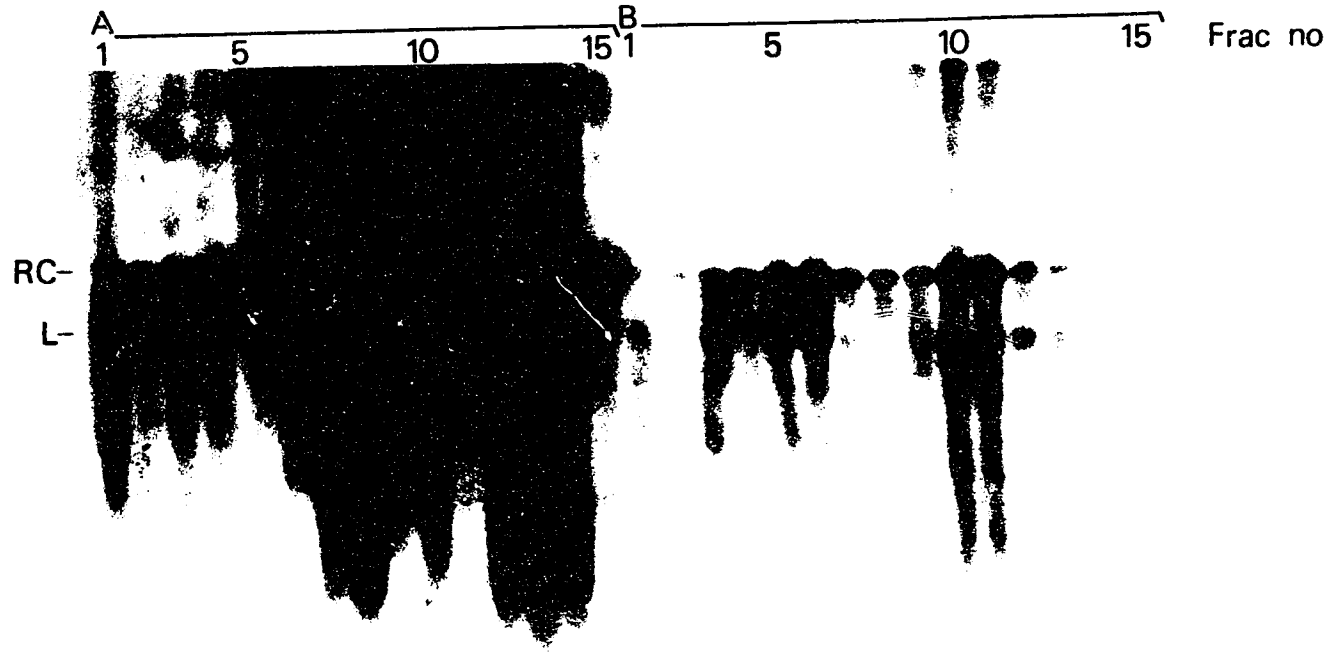
checked the viral nucleic acids in the same preparation of replicative core particles and the result of the Southern blot shows a considerable decrease in the amount of viral DNA as a result of treatment with ddDAPR (Fig. 4.5).

4.3 Attempts to Identify the Terminal Protein of Duck Hepatitis B virus.

The demonstration of a protein bound to the complete DNA strand of HBV virions was first reported by Gerlich and Robinson (1980). This observation was expanded by Molnar-Kimber *et al.* (1983) when they demonstrated the binding of the protein to the 5' end of nascent minus strand DNA in DHBV replicative cores. When I began my studies, there was little known about the size or origin of this terminal protein. However, recent studies by Bosch *et al.* (1988) have provided evidence that the terminal protein is derived from the viral polymerase. We have proposed the mechanism that purine 2',3'-dideoxynucleosides specifically inhibit viral DNA synthesis by covalently bonding to the terminal protein (see discussion). If this hypothesis is true, then we should be able to specifically label the terminal protein with α-labelled ^{35}S-ddATP. This marker was used since at the time of these studies it was the only α-labelled dideoxynucleotide available. These studies were complicated by the fact that while nucleosides cross cell membranes easily, nucleotides do not. Therefore we used cell lysates to attempt to

Figure 4.5.

Southern blot hybridization of viral DNA from replicative core particles isolated from livers of placebo (Panel A) or ddDAPR (Panel B) treated ducks. The numbers across the top and the letters on the left indicate sucrose gradient fractions and viral DNA forms as described in the legend to figure 4.4.


A
B
1
5
10
15
1
5
10
15
Frac no
RC–
L–

demonstrate covalently binding of the labelled nucleotide to the terminal protein.

The results of incubating [α-^{35}S]ddATP with cytoplasmic and nuclear extracts from DHBV infected and uninfected hepatocytes are shown in figure 4.6. In these studies, which were repeated three times, we have consistently found that the labelled nucleotide binds to a protein of approximately 14 kDa in the cell extracts of infected hepatocytes. This protein was not found in incubation mixtures prepared from uninfected hepatocytes. These results are very preliminary and suffer from the lack of an appropriately labelled pyrimidine dideoxynucleotide which should be used as a control to determine the specificity of the binding. We placed a custom order for α-labelled ^{35}S-ddCTP and ^{35}S-ddGTP, however the chemists at Dupont have been unable to synthesize these analogues at present.

In the meantime, I have attempted to determine the molecular weight of terminal protein. My approach was to purify the terminal protein-DNA complex (TPDC), iodinate the protein, remove the DNA by enzymatic digestion, and identify the iodinated protein by gel electrophoresis. First, I iodinated the TPDC from purified DHBV virions and examined two aliquots of the preparation, one with and one without proteinase K digestion, on SDS-agarose gels to confirm the presence of the terminal protein on DHBV DNA genome (Fig. 4.7). There was a change in mobility of the genomic DNA

after proteinase K digestion. The protein of the TPDC was then labelled with ^{125}I and the labelled product was digested with either DNase I or Micrococcal endonuclease to remove the DNA from the TPDC before electrophoresis of the protein. I have tried a number of electrophoresis systems to detect the labelled terminal protein. Only with SDS-Urea-PAGE gel electrophoresis was I able to show a unique protein band, as shown in figure 4.8. In a 12% PAGE gel containing SDS (0.1%) and urea (8 M), there was a band detectable after the digestion with DNase I (Fig. 4.8, column b) or Micrococcal endonuclease (Fig. 4.8, column c). No corresponding band was detected with the undigested TPDC (Fig. 4.8, column a). The molecular weight of this protein was approximately 10 kDa. When the TPDC was treated with proteinase K, all of the iodinated protein disappeared (Fig. 4.8, column d). These results were complicated by a large amount of iodinated material (presumably protein since it disappears with proteinase K digestion) at the origins of both the stacking and separation gels. The nature of this protein is unclear, but it could be an aggregate of protein, or protein-DNA if the digestion with DNase I or nuclease were not complete.

Figure 4.6.

SDS-PAGE gel electrophoresis and autoradiography of the proteins labelled in a "binding assay" with [^{35}S]ddATP. Column a shows the proteins from a reaction containing an uninfected duck hepatocyte lysate. Column b shows the proteins from a reaction containing a DHBV-infected duck hepatocyte lysate. The arrow indicates a band which may represent a viral protein bound to [^{35}S]ddATP. Numbers on the left indicate the positions of molecular weight marker proteins.

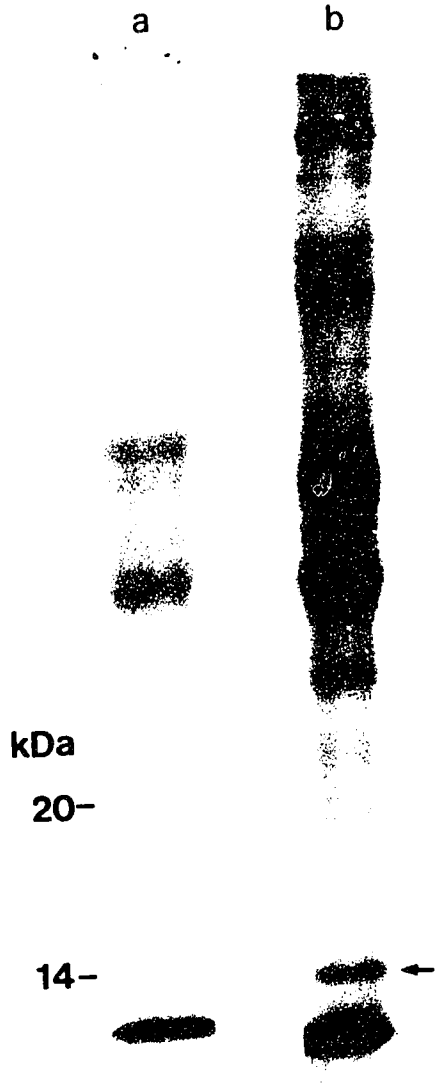

a
b
kDa
20–
14–

Figure 4.7.

Southern blot hybridization of the terminal protein-DNA complex (TPDC). TPDC was electrophoresed on 1.5% agarose containing 0.1% SDS with no treatment (lane b) or after treatment with proteinase K (50 µg/ml, for 30 min at 37° C) (lane c). A mixture of molecular weight markers was run in lane a and molecular weights corresponding to these markers are indicated in kilobases on the left.

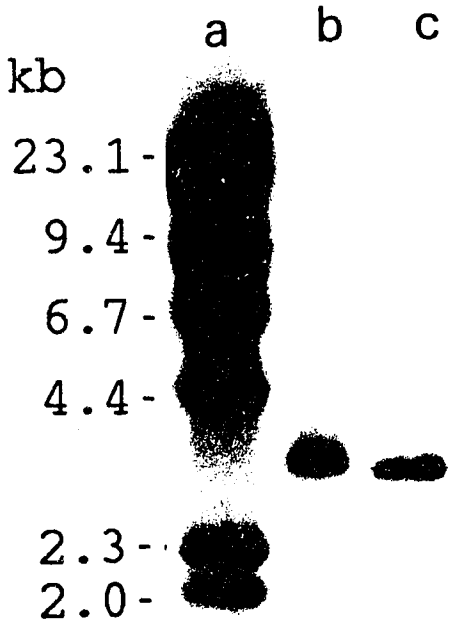
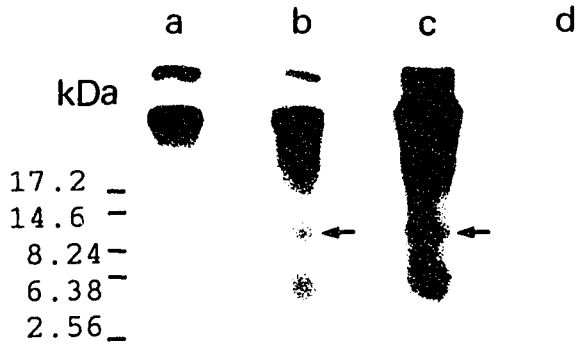

a
b
c
kb
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-

Figure 4.8.

SDS-Urea-PAGE gel electrophoresis and autoradiography of the [^{125}I]-labelled terminal protein-DNA complex (TPDC). Lane a illustrates the proteins from [^{125}I]-labelled TPDC before enzymatic digestion. Lanes b, c, and d show the proteins from [^{125}I]-labelled TPDC after digestion with (b) DNase I, or (c) Micrococcal nuclease, or (d) proteinase K. Numbers on the left indicate the positions of molecular weight (in kilodalton) protein markers. Arrows indicate the band which may be the terminal protein.


a
b
c
d
kDa
17.2
14.6
8.24
6.38
2.56

DISCUSSION

DHBV replication is strongly inhibited by purine but not by pyrimidine 2',3'-dideoxynucleosides. This was an interesting finding and one which we could exploit to try to determine the unique effect of the purine analogues. Firstly, I examined the competition and chain termination effects of ddGTP and ddCTP on viral DNA polymerase and reverse transcriptase activities in order to test whether these two dideoxynucleotide analogues can effectively compete with their corresponding deoxynucleotides to terminate DNA chain elongation. My results show that both ddCTP and ddGTP are relatively weak chain terminators under the condition I used in these experiments. These effects of ddGTP were inadequate to account for the marked inhibitory effect of ddG on virus clearance in cell cultures or the rapid clearance of the virus from sera of ducks treated *in vivo* (Suzuki *et al.*, 1988, Lee *et al.*, 1989). The chain termination effects of ddGTP and ddCTP in my studies are in reasonable agreement with the results of Lofgren *et al.* (1989). In their study, the IC_{50} for reverse transcriptase was 7 μM for ddCTP and 1 μM for ddGTP. The IC_{50} values obtained in my studies were 6.9 μM and 3.2 μM for ddCTP and ddGTP, respectively. Lofgren *et al.* (1989) also reported that ddTTP had an IC_{50} of 0.4 μM for DNA polymerase and 4.1 μM for reverse transcriptase. From my results and the results reported by Lofgren *et al.*(1989), it is not possible to attribute the marked difference between

the abilities of the purine and the pyrimidine analogues to inhibit hepadnavirus replication to differential effects on either DNA polymerase or reverse transcriptase. We found that ddT was completely ineffective as an inhibitor of DHBV replication, yet ddTTP is comparable to ddGTP in its ability to inhibit DNA polymerase and reverse transcriptase (Lofgren *et al.*, 1989).

A possible explanation for the different effects of the purine and pyrimidine dideoxynucleosides could be differences in the rates of phosphorylation to the mono, di and triphosphate nucleotides. The metabolism of these analogues in cell cultures has been studied and reported at the Molecular Biology of Hepatitis B Virus Meeting in San Diego (Kitos and Tyrrell, 1990). These studies demonstrated that differential rates of phosphorylation were not sufficient to explain the marked differences observed in the inhibition of DHBV.

The effects of ddDAPR and ddC on viral replication have also been compared by measuring the DNA and RNA in the replicative core particles. The results have demonstrated that ddDAPR effectively inhibits accumulation of core particles containing viral DNA. Although there was a decrease in the amount of pregenomic RNA with ddDAPR treatment, the decrease was not as great as the decrease in viral DNA. This suggested that ddDAPR had a direct effect on DNA synthesis which probably led to an indirect effect on

pregenomic RNA synthesis. In contrast, treatment with ddC did not show any inhibitory effects on the accumulation of core particles containing either viral DNA or pregenomic RNA.

I believe that treatment with ddDAPR should also cause a decrease in the production of the total number of replicative core particles in duck livers. The decreases in viral DNA and pregenomic RNA that are evident in the Southern and Northern blots of replicative core particles are in part consequence of a decreased amount of replicative core particles in ddDAPR treated ducks. Unfortunately, I did not have good antisera against core particles which could have been used to standardize the amounts of core particles used for these studies Nevertheless, the results of the Northern blot indicated that a much lower reduction occurs in pregenomic RNA than in viral DNA as analyzed on the Southern blot. It is possible that the decrease in pregenomic RNA reflects the decrease in total replicative core particles, which in turn is a consequence of the decrease in cccDNA in the nuclei of the hepatocytes (see Chapter 3). The greater decrease in viral DNA could be accounted for by a combined decreases in core particles and inhibition of viral DNA synthesis within the particles.

The analysis of DNA in the replicative core particles from ddDAPR treated animals showed that almost all viral DNA chains were blocked at an early stage of DNA synthesis, although there were still pregenomic RNA templates. The fact

that the effect can be seen in the endogenous labelling reaction suggests that the blockage of viral DNA synthesis by ddDAPR must be the result of very strong binding of the inhibitor to its target since any inhibitor not tightly bound should be removed during the preparation of the replicative cores. The binding of the inhibitor to its target probably involves covalent bonding although the possibility that some other form of irreversible inhibition cannot yet be excluded. The fact that some dideoxynucleoside analogues inhibit DHBV replication while others do not may enable us to test the hypothesis that the mechanism of this inhibition involves covalent or tight binding of an inhibitor to its target.

Based on the current knowledge of hepadnavirus replication, we have carefully looked at the steps which may possibly serve as specific targets for inhibition by 2',3'-dideoxyguanosine during the viral replication. During hepadnavirus replication, the first step is repair of the single stranded gap in the plus strand of DNA before the viral genome is converted into cccDNA. The frequency of dGs in this gap is not very high. In fact, dC is the most frequently occurring nucleotide in the last 100 nucleotides of the plus stand of the DHBV genome. Thus, it is unlikely that the step of repairing the gap is the unique target for dideoxyguanosine analogues. After the partially double-stranded genome is converted into cccDNA, pregenomic RNA is produced from the cccDNA by RNA polymerase II. The

pregenomic RNA serves as the template for minus strand DNA synthesis in the replicative cores. This synthesis is primed by a protein and the initial nucleotide bound to the terminal protein is deoxyguanoylic acid in DHBV (Fig. 1.3). As I mentioned in the introduction, deoxyguanylic acid appears to be conserved as the first nucleotide in the minus strand DNA of all hepadnaviruses sequenced to date. It is not unreasonable to expect that this deoxyguanylic acid or deoxyquanosine triphosphate is very important in initiation of DNA synthesis by the viral polymerase, and that it may also be the site for specific inhibition by dideoxyguanosine triphosphate analogues. Inhibition of DNA synthesis can also occur during chain elongation. Both purine and pyrimidine 2',3'-dideoxynucleotides have the potential to block chain elongation. The efficiency of inhibition should be dependent upon the ability of a dideoxynucleotide analogue to compete with the natural nucleotides in the elongation process. This competition probably occurs, however, my studies on these effects of ddGTP and ddCTP on the viral DNA polymerase activity failed to show a significant differential effect. Another interesting potential site for inhibition is the initiation of plus strand DNA. This step is a complex process and involves two template switches (an oligoribonucleotide primer switch and an intramolecular switch, see Fig. 1.2). However it is unlikely that this is the specific site of action of purine 2',3'-dideoxynucleosides since no site seems specific for ddG or

ddA. Further, the results of my studies show that the DNA synthesis must be blocked very early since there is inhibition of both negative and positive strand. Inhibiton at this late step would have shown greater inhibition of the plus stand than the minus strand.

Considering all possibilities, I suggest that the first nucleotide linked to the terminal protein is most likely to be the specific target for inhibition by 2',3'-dideoxyguanosine analogues. Therefore I have proposed that the 2',3'-dideoxyguanosine analogues compete with the initial deoxyguanosine nucleotide for linkage to the terminal protein and inhibit DHBV replication at early stage.

Other purine 2',3'-dideoxynucleosides such as ddA and ddI have shown some inhibitory effects on DHBV replication in hepatocyte cultures. I hypothesize that this is a result of some "wobble" in the purine base that binds to the terminal protein. For example, a $C \cdot A^+$ wobble base pair could be formed (Hunter, *et al.* 1986). Therefore, if the inhibition of DHBV replication by purine 2',3'-dideoxynucleosides does occur at the initiation step of minus strand DNA synthesis, purine dideoxynucleoside analogues other than ddG and ddDAPR could also inhibit viral replication, but probably with lesser efficiencies. The results of my studies done *in vitro* (Fig.2.3) and *in vivo* (Fig. 2.4) seem to match this hypothesis. Other evidence supporting this possibility can be found in the report of Seeger *et al.*(1990). They have

found that the synthesis of the minus strand DNA could be initiated at sites encoding either dG or dA, but not at sites encoding a pyrimidine deoxynucleotide. This indicates that the viral enzyme has a preference for the purine deoxynucleotides in initiating the synthesis of the minus strand. With this data and data from our *in vitro* studies, we were encouraged to try to label the terminal protein with α-labelled ^{35}S-ddATP since this was the only α-labelled purine 2',3'-dideoxynucleoside available at the time of these studies.

Identification of the terminal protein may be necessary in order to obtain direct evidence to support our hypothesis. However, this identification is not easy because, in contrast to the situation with adenoviruses, there is no *in vitro* replication system for hepadnaviruses. Our idea for a "binding assay" was based on a hope that the reaction with ddGTP or ddATP, similar to that which normally occurs with dGTP in initiation of the synthesis of minus strand DNA, could take place *in vitro*. We therefore thought that [α^{35}S]ddATP might bind the terminal protein in lysates from infected hepatocytes. As I have shown in the binding assay, [α^{35}S]ddATP binds to a protein in DHBV-infected hepatocyte lysates which bands at approximately 14 kDa in SDS-PAGE. There was no evidence for such a labelled protein in reactions with extracts from uninfected hepatocytes. We recognize that these studies are preliminary and the more

work with α-labelled ddGTP and ddCTP will be necessary to lend further support to our hypothesis.

The DNase and nuclease digested products of [^{125}I] labelled TPDC also showed a protein band of a lower molecular weight (approximately 8-10 kDa on an SDS-urea PAGE gel). These observations suggest that a low molecular weight protein binds to the negative strand of DHBV DNA or is covalently bonded to α-labelled ^{35}S-ddATP. Stronger evidence for binding might be obtained by labelling the terminal protein with [α^{35}S]-ddGTP and immunoprecipitating the protein with a specific antiserum.

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE PROSPECTS

The antiviral activity of some 2',3'-dideoxynucleosides against hepadnaviruses has been demonstrated using a DHBV model. The purine 2',3'-dideoxynucleosides are strong inhibitors of DHBV replication in DHBV infected primary hepatocyte cultures, with 2',3'-dideoxyguanosine and an analogue, ddDAPR, being the most effective of those I tested. The pyrimidine 2',3'-dideoxynucleoside analogues tested in my studies failed to show a strong inhibition of DHBV replication *in vitro*. This selective sensitivity of DHBV to the purine 2',3'-dideoxynucleosides suggests a unique mechanism of action for these active compounds.

Purine 2',3'-dideoxynucleosides demonstrated potent antiviral activity *in vivo* as well as *in vitro*. This observation was very encouraging and it strengthened my belief that these compounds have significant potential for the treatment of acute or chronic hepadnavirus infections. Once an effective agent has been found in an animal system, a great deal more work still needs to be completed before such an agent can be tried in humans, however. To list some of the studies that would need to be completed in this case, for example, we should show that the agents effective in the avian model will also work in the mammalian models, define the steps in uptake and metabolism of the compound, determine

its mechanism of action, and toxicity. It would also be desirable to gain a better understanding of the molecular biology of the virus and its replication in order to better predict whether or not long term therapy could "cure" the chronic carrier. I have chosen to concentrate on three aspects of the project for my thesis work. I have carried out some of the preliminary screening of potentially interesting compounds, measured the half lives of some of the viral replicative intermediates using these antiviral agents as important tools, and finally, attempted to test my hypothesis concerning the unique mechanism of action of purine 2',3'-dideoxynucleosides.

Firstly, I want to discuss the antiviral screening studies. This system is relatively inexpensive to use, and reasonably rapid (3-4 weeks). More work will be required to ascertain whether or not activity in the duck model is predictive of activity in the human system. In preliminary studies using hepatoma cell lines transfected with HBV, the activities of a number of compounds have not correlated very well with their activities in DHBV-infected hepatocytes (Dorothy Tovell, Dr. Tyrrell's lab. and personal communication, Brent Korba, NIH). It is not possible at this time to say whether these differences can be attributed to differences between duck and human cells or between primary hepatocytes (non transformed cells) and cells of a hepatoma line.

Secondly, I have utilized the potent *in vivo* antiviral activities of these compounds to obtain new information about the half lives of the virion in serum and the cccDNA in hepatocytes. I measured the rate of decrease of DHBV DNA in sera of treated ducks and found that the half life of DHBV DNA in sera was relatively short, namely from 11 to 14 hours. Not surprisingly, the half life of cccDNA in hepatocytes was considerably longer. This information will be useful in designing experiments in which we will try to "cure" a chronically infected animal.

Thirdly, I proposed a mechanism of inhibition of DHBV replication by ddDAPR based on the results of my *in vitro* studies and on the publication of the replicative pathway of hepadnaviruses (Summers and Mason, 1982). I hypothesized that 2',3'-dideoxyguanosine analogues compete with dGTP for binding to the terminal protein. The dideoxynucleotide analogue linked to the terminal protein cannot be removed and therefore inhibition of DHBV DNA replication by ddDAPR or ddG occurs at the initial step of the minus strand synthesis. I have attempted to obtain evidence regarding this hypothesis using three approaches. Firstly, I have shown that under the condition I used, ddGTP and ddCTP are not effective competitors of their corresponding deoxynucleotides with DHBV DNA polymerase or reverse transcriptase. It is hard to explain the different inhibitory effects between ddG and ddC by chain termination. It is possible that other specific

target for 2',3'-dideoxyguanosine analogues is involved. Secondly, accumulation of core particles containing viral DNA is largely inhibited in ddDAPR-treated ducks. In addition, pregenomic RNA is less affected by the treatment with ddDAPR. These results suggest that the inhibition of DHBV replication occurs at the stage of viral DNA synthesis in the replicative core complex. Examination of the occurrence of deoxyguanylic acid in various hepadnaviral DNA revealed that the first nucleotide on the minus strand DNA of the viral genome is conserved and is possibly the specific target. On this basis, I have tried to demonstrate binding of [α^{35}S]ddATP to the viral terminal protein. Such a demonstration would provide more direct evidence for our hypothesis. In these studies, I have obtained preliminary evidence that α-labelled ^{35}S-ddATP binds to a protein in infected cells, and that such a binding reaction cannot be detected in uninfected cells. More studies will be required to determine whether this is the terminal protein.

Fairly early in my thesis work, I treated DHBV-infected ducks with ddDAPR at 10 mg/kg bid for 7 months. The virus was rapidly cleared from the sera as tested by dot hybridization. However, within 3 weeks after cessation of the therapy, all three ducks showed a relapse as evidenced by reappearance of virus in the serum. Our more recent results show clearly that the dose used in these studies was sub-optimal and that the drug must be given every 6-8 hours. An

attempt to cure animals is now being undertaken based on this more complete information. Furthermore, it may be worthwhile to compare the response in congenitally infected and infected-at-birth animals since help from the immune system may be important in achieving a cure. Immune help is more likely to be present in infected-at-birth animals than in congenitally infected animals. In addition elimination of the virus from sera might better be assayed by a more sensitive means such as PCR instead of by simple dot blots.

Finally, I believe that chronic hepatitis B infection will some day be cured by antiviral therapy. I am not sure that the "best" antiviral agents will be the ones I have discussed in my thesis. Other effective agents are likely to be discovered. It was most encouraging to me to discover that hepadnaviruses have "relatively short half lives" in serum (at least in the duck model). However, cccDNA in the hepatocyte appears to be very stable. More novel approaches to shorten the half-life of cccDNA or to selectively eliminate infected cells will have to be found to "cure" chronic carriers.

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